#44 GDD

Docket No.: 104385-140 PATENT/OFFICIAL

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :

D. Clark Bennett et al. :

Serial No. 08/722,659 : Group Art Unit: 1644

Filed: 27 September 1996 : Examiner: A. DeCloux

For: USE OF HEPARINASE TO DECREASE INFLAMMATORY RESPONSE

APPEAL BRIEF UNDER 37 C.F.R. § 1.192

Honorable Commissioner of
Patents and Trademarks
Washington, D.C. 20231

Sir:

This is an Appeal Brief from the final rejection of May 31, 2002, rejecting claims 1-7, 18, and 19, and Notice of Appeal filed on September 3, 2002. This Brief is being filed in triplicate.

I. REAL PARTY IN INTEREST

The inventors assigned the present application to IBEX Technologies, Inc. by virtue of an assignment recorded in the U.S. Patent Office on June 6, 1997, at Reel 8623, Frame 0914. The present application was then assigned from IBEX Technologies R&D, Inc., which was transferred and assigned to BioMarin Enzymes Inc. by virtue of an assignment recorded in the U.S. Patent Office on November 1, 2001, and attached hereto as Appendix A. Accordingly, the Real Party in Interest is BioMarin Enzymes Inc.

II. RELATED APPEALS AND INTERFERENCES

The Appellants, the Appellants' legal representatives, and the Assignee are not aware of any pending appeals or interferences that would directly or indirectly affect or have a bearing on the Board's decision in this appeal.

III. STATUS OF THE CLAIMS

Claims 1-7 and 18-19 are pending in the application. Claims 8-17 were canceled from the application during prosecution. Claims 1-7 and 18-19 are rejected. No claims are allowed.

IV. STATUS OF AMENDMENTS

The Response with Declarations filed August 8, 2002, was not entered by the Examiner. The claims were last amended in the Response filed March 18, 2002. A copy of the claims, as they now stand, is provided in Appendix B.

V. SUMMARY OF THE INVENTION

Inflammation of tissues in the body is the local response to cellular injury, such as by trauma, damage, infection, etc. of the cells. Detrimental inflammatory response may involve accumulation of leukocytes within a tissue and can include ischemia/reperfusion injury, such as as that followed by myocardial infarction. Ischemia is defined as a "decrease in the blood supply to a bodily organ, tissue, or part caused by constriction or obstruction of the blood vessels." (Appendix C.) Reperfusion is defined as the "restoration of blood flow to an organ or tissue that has had its blood supply cut off, as after a heart attack." (Appendix D.)

The inflammatory response occurs by recruitment of leukocytes, such as neutrophils, to the damaged or infected tissue. Leukocyte recruitment involves a cascade of cellular events, beginning with activation of vascular endothelium ("activated endothelium") by damaged or infected tissue adjacent to the endothelium resulting in enhanced adhesion of leukocytes to the endothelium cells. Adhesion of the leukocytes to the endothelium then allows for transendothelial migration ("transmigration" or "extravasation") by bound leukocytes, such as neutrophils, into the damaged cells. A neutrophil is defined as a "large, granular leukocyte that will stain with neutral dyes and eosin (a red, fluorescent dye) and which has a multi-lobed, irregular nucleus." (Appendix E.) Chemotactic factors have been found to induce neutrophil adherence to the activated endothelium and transmigration thereof. This transmigration, for example, is illustrated in the following figure from Frangogiannis et al., "The Inflammatory Response in Myocardial Infarction," *Cardiovascular Research* 53:31-147 (2002), provided in the Response filed March 18, 2002 and attached hereto as Appendix F:

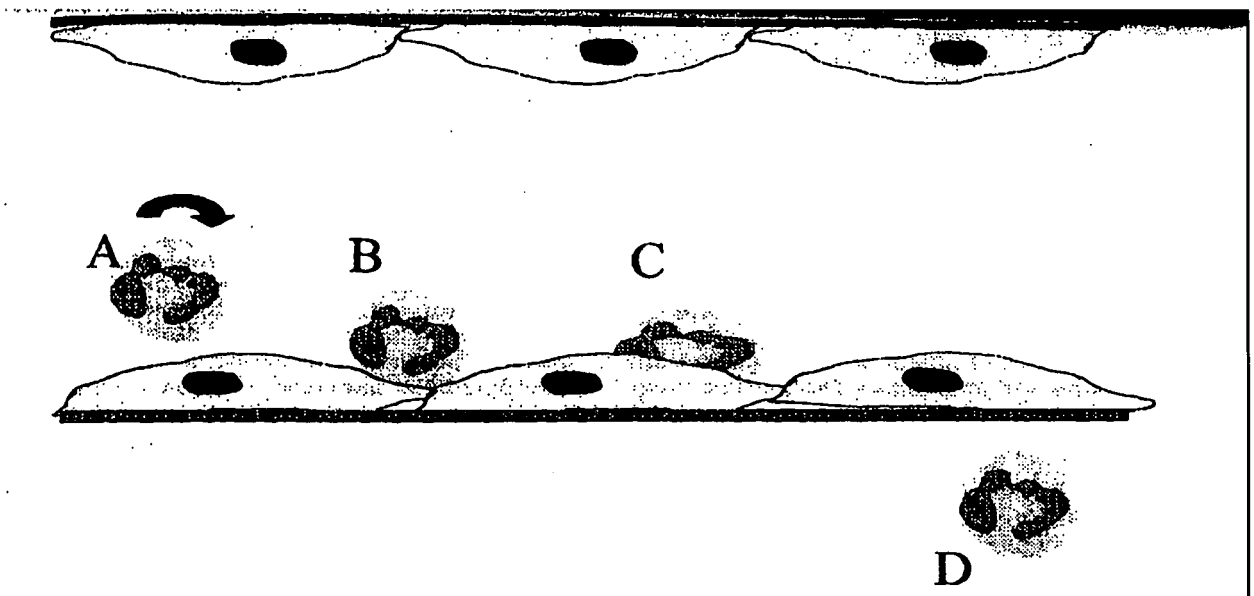

Fig. 1. Diagram illustrating the sequential steps of the adhesion cascade regulating neutrophil localization in post-capillary venules during the early stages of acute inflammation. (A) After the inflammatory stimulus is applied, leukocytes roll along the post-capillary venules at velocities distinctly below that of flowing blood. (B) Some rolling cells can be seen to arrest and after a few minutes change shape (C) in apparent response to local chemotactic stimuli. Extravasation into the extravascular tissue follows (D). Each of these steps requires either upregulation or activation of distinct sets of adhesion molecules.

The secretion of chemotactic factors, such as thrombin, histamine, selectins, cytokines, chemoattractants, etc., serve to activate the endothelial cell surface enabling the leukocyte to roll along the endothelium, interact with these secreted molecules, and adhere to the endothelium. (*See* Specification, pages 2-4.) Transmigration or extravasation of the adhered leukocytes follows, as illustrated in the following figure from Frangogiannis:

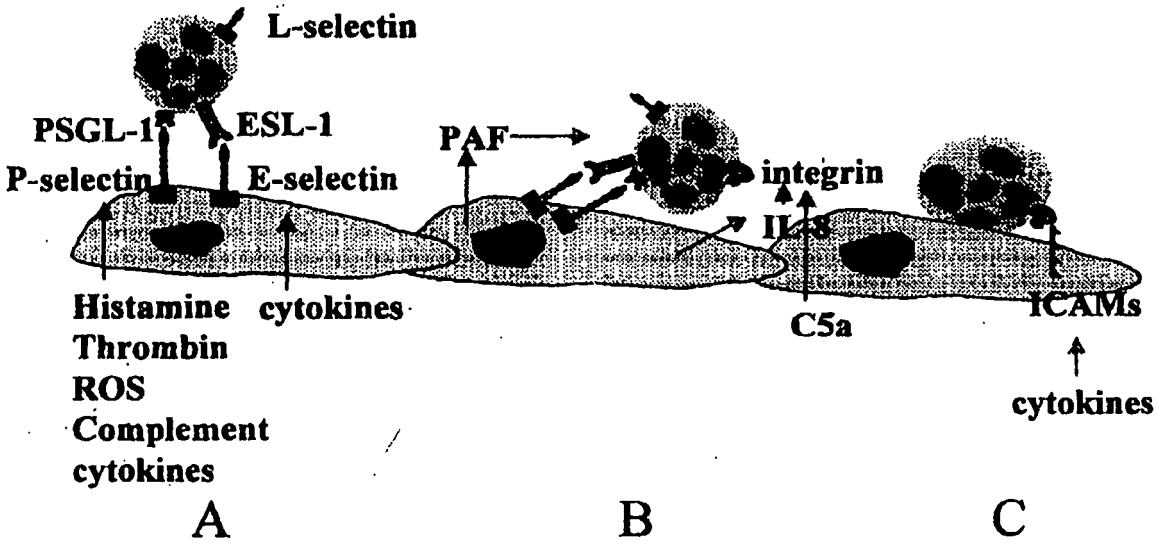

Fig. 2. Endothelial–neutrophil interactions leading to neutrophil transmigration into the injured myocardium. (A) The initial tethering of neutrophils to the endothelial cell surface is mediated by the selectins. (B) This enables the leukocyte to roll along the venular wall and to 'sense' activating factors (such as IL-8 and C5a). These interactions lead to neutrophil integrin activation. (C) Firm adhesion of the leukocyte is mediated through binding of neutrophil integrins to members of the immunoglobulin superfamily expressed in stimulated endothelial cells. Abbreviations: P-Selectin Glycoprotein Ligand-1, PSGL-1; E-Selectin Ligand-1, ESL-1; Reactive oxygen species, ROS; Platelet Activating Factor, PAF; Interleukin-8, IL-8; Intercellular Adhesion Molecule, ICAM.

Therefore, neutrophil transmigration across the vascular endothelium and basement membrane to the site of tissue injury is one of the first steps in an inflammatory response.

Heparinase is an enzyme that cleaves at glucosamine (1-4) hexuronic acid linkage sites. Appellants have found that heparinase is useful to disrupt the steps that lead to a localized inflammatory response. In particular, appellants have found that the intravascular administration of heparinase decreases neutrophil transmigration through the activated endothelium and basement membrane. Appellants have found that this decrease in transmigration results in a reduced localized inflammatory response, for example, from ischemia or reperfusion injury.

VI. ISSUE

Whether claims 1-7 and 18-19 are unpatentable under 35 U.S.C. §102(e) or (f) as being anticipated over U.S. Patent No. 5,997,863 issued to Zimmermannn et al. ("the Zimmermannn patent").

VII. GROUPING OF CLAIMS

Claims 1-7 and 18-19 stand or fall together.

VIII. ARGUMENT

Claims 1-7 and 18-19 were rejected under 35 U.S.C. 102(e) or (f) as being anticipated by U.S. Patent No. 5,997,863 issued to Zimmermannn et al. ("Zimmermann").

With respect to the rejection under 35 U.S.C. 102(e), the Examiner contends that Zimmermannn teaches a method of treating ischemia in a rabbit hind limb ischemic model by administering heparinase 1 as set forth at Example 8, column 17, line 62 through column 18, line 34. (*See* Office Action mailed August 1, 2000 at 2.) The Examiner further states that Zimmermann discloses that the administration of heparinase releases heparin binding growth factors and degrading components of the extracellular matrix, which facilitates the mobility of cytokines, chemoatttractants and cells (col. 6, lines 25-59). Moreover, it is stated that the Zimmermann patent describes that wound healing is divided into three phases, inflammation, proliferation, and remodeling. The Examiner emphasizes that Zimmermann describes that during inflammation, the blood borne cells infiltrate the wound site and release mediating factors (col. 2, lines 56-67). The Examiner impermissibly relies on the instant specification to teach that ischemia induces inflammatory responses such as migration of neutrophils across the

connective tissue, extravasation of plasma, and other blood and cellular components. (Office Action mailed August 1, 2000 at 2.) The Examiner concludes that the claims are anticipated because "the method of treating ischemia by administering heparinase taught by the '863 patent would also decrease the localized inflammatory responses that result from ischemia." (Office Action mailed August 1, 2000 at 2-3.)

With respect to the rejection under 35 U.S.C. 102(f), the Office Actions do not elucidate the positions taken with respect to this alternative rejection.

Anticipation under 35 U.S.C. §102 requires that each and every element of the claimed invention be disclosed either expressly or inherently in a single prior art. *In re* Robertson, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950 (Fed. Cir. 1999). The single reference must describe and enable all elements of the claimed invention to establish that the subject matter already existed in the prior art and that "its existence was recognized by persons of ordinary skill in the field of the invention." *Crown Operations International, Ltd. V. Solutia Inc.*, 289 F.3d 1367, 1375, 62 USPQ2d 1917, 1921 (Fed. Cir. 2002). Thus, in order to anticipate the instant invention, Zimmermann must disclose each and every element of the claimed invention, either expressly or inherently, to have placed a person of ordinary skill in the field of the invention in possession of it. *See, e.g., In re Spada*, 911 F.2d 705, 708, 15 USPQD2d 1655, 1657 (Fed. Cir. 1990). Because the Zimmermannn patent fails to expressly or inherently disclose each of the elements recited in claims 1-7 and 18-19, the Zimmermann patent is not anticipatory. Reversal of this rejection is respectfully requested.

The claims of the instant invention are directed to a method of decreasing localized inflammatory responses arising from an ischemia/reperfusion injury in a tissue of a patient

comprising intravascularly administering to said patient heparinase enzyme in an effective amount sufficient to decrease neutrophil transmigration through activated endothelium and basement membrane of said vasculature which decreases said localized inflammatory response arising from an ischemia/reperfusion injury.

Zimmermann describes a process of enhanced wound healing by administration of heparinase. However, the Zimmermann patent first does not teach that induces ischemia induces an inflammatory response and second, does not teach that intravascular administration of heparinase results in a reduced localized inflammatory response arising from an ischemia/reperfusion injury by decreasing neutrophil transmigration through activated endothelium and basement membrane of the vasculature.

A. Zimmermann Teaches Heparinase Enhances Neutrophil Transmigration Which Is Contrary To The Claimed Invention

As described in the specification at pages 1-8 and in the review article, Singer and Clark, "Cutaneous Wound Healing," Mechanisms of Disease, 341(10):738-746 (1999)(cited in Response filed March 18, 2002), inflammation involves the adherence of neutrophils to an activated extracellular matrix followed by the infiltration or transmigration of neutrophils into the wounded area. The claims are directed to reducing the infiltration or "transmigration" of neutrophils through the activated cellular matrix and basement membrane by the administration of heparinase.

Zimmermann addresses a different mechanism of action by the use of heparinase to enhance wound healing. Specifically, the Zimmermann patent states that the heparinase "releases heparan sulfate fragments and heparin binding growth factors from the extracellular matrix, thereby increasing their availability to the adjacent cell surface receptors, and increases

the mobility of molecules such as chemoattractants, growth factors, and cells through the extracellular matrix." (Col. 5, lines 50-57; emphasis supplied.) Therefore, Zimmermann suggests that transmigration of cells through the extracellular matrix is enhanced by the administration of heparinase. This is contrary to the claimed invention, which recites decreasing transmigration of neutrophils through the matrix. Therefore, unlike the claimed invention, Zimmermann teaches an increase in transmigration of cells through the extracellular matrix. An increase in transmigration leads to increased inflammation, which is the very antithesis of the claimed method of reducing localized inflammation.

B. Wound Healing As Described by The Zimmermann Patent Cannot Be Equated To Reducing Inflammation

Zimmermann teaches that wound healing involves at least three different phases: inflammation, proliferation, and remodeling. The patent describes that during inflammation, blood borne cells infiltrate the wound site and that neutrophils may assist by secreting degradative enzymes, elastase and collagenase, to enhance the passage of cells through the basement membranes. (*See, e.g.,* col. 2, lines 62-65 and col. 3, lines 7-9.) The proliferative phase, as described in the Zimmermann patent, involves the migration of keratinocyte and epidermal cells to the wound site. (Col. 3, lines 10-17.) Finally, tissue remodeling is described as involving the secretion of matrix components, which serve as a scaffold for cellular migration and tissue support. Similarly, Singer and Clark confirms the distinctions between inflammation, tissue formation, and tissue remodeling as distinct phases of wound healing.

Since inflammation is one necessary step in the wound healing process, the Zimmermann patent teaches away from the present invention. The Zimmermann patent is directed to enhanced wound healing by administration of heparinase, which would require that each of the steps of

inflammation, proliferation, and remodeling be undertaken to achieve wound healing. As such, inflammation is a required step in the wound healing process. Therefore, unlike the claimed invention, the Zimmermann patent does not teach reducing inflammation. Instead, it requires that inflammation, as part of the wound healing process, occur followed by proliferation and remodeling. Accordingly, Zimmermann does not teach a method of reducing a localized inflammatory response as recited in the claim.

C. Enhanced Cell Proliferation and Revascularization As Described by Zimmermann Does Not Teach the Claimed Invention

Zimmermann addresses the use of heparinase to enhance cell proliferation and revascularization, each of which are distinct phases of wound healing from the inflammation phase. Zimmermann describes that the extracellular matrix ("ECM") is a multi-component structure synthesized by and surrounding cell types, including endothelial cells. (Col. 2, lines 32-34.) The ECM comprises proteoglycans, such as heparan sulfate, present on the cell surface, where they act as cytokine receptors. (Col. 2, lines 35-37 and col. 6, lines 28-32.) Growth factors are sequestered in the ECM, and have been implicated in cell proliferation. (Col. 2, lines37-40 and col. 1, lines 8-10 and 30-34.) Zimmermann describes that the heparinases are administered to degrade the heparan sulfate components of the ECM allowing the heparin binding growth factors to migrate to adjacent cells, which are then implicated in enhanced cell proliferation. According to the Zimmermann patent,

> heparinase . . . modulate[s] the interactions involved in cell proliferation and migration by i) releasing heparin binding growth factors and molecules from the extracellular matrix, thereby increasing their availability to adjacent cells for the stimulation of proliferation and migration, ii) degrading components of the extracellular matrix, thereby facilitating the mobility of cytokines, chemoattractants and cells, iii) removing chondroitin sulfate from cell surfaces, thereby increasing access to cell surface receptors and iv) inhibiting the proliferative response of cells to growth factors by removing the heparan sulfate component of their growth factor receptor complex. (Col. 6, lines 34-48.)

Therefore, the Zimmermann patent teaches that heparinase modulates cell proliferation in the wound healing process through a mechanism involving growth factors.

In addition, the Examiner relies on Example 8 of Zimmermann, "Evaluation of Local Administration of Heparinase to Enhance Revascularization," to support the rejection. Example 8 describes the induction of ischemia in the hind limbs of rabbits followed by administration of heparinase. It was noted that heparinase increased the blood pressure ratio indicating blood vessel formation. (Col. 18, lines 9-25.) Zimmermann concluded that heparinase was useful to accelerate tissue repair in humans. (Col. 18, lines 26-30.)

However, the disclosure of the use of heparinase to modulate cell proliferation, and enhance revascularization are separate steps in the wound healing process relative to the inflammation process. These processes are dependent upon different factors than that required by inflammation. Therefore, Zimmermann does not teach each and every element of the claimed invention.

In fact, Zimmermann does not teach that ischemia or reperfusion injuries may result in an inflammatory response. Instead, the Examiner impermissibly relies on the Appellants' own specification to teach that ischemia causes a localized inflammatory response. Indeed, Appellants' own specification cannot be applied as prior art under 35 U.S.C. 102.

Moreover, Zimmermann does not describe the reduction of localized inflammation. Its focus is primarily on the effects of heparinase to enhance wound healing, and emphasizes the modulation of tissue formation and revascularization, not inflammation. Additionally, Zimmermann never describes that heparinase acts to decrease neutrophil transmigration through the activated endothelium and basement membrane. Accordingly, the Zimmermann patent does not teach the claimed invention because it fails to describe each and every element of the claimed invention.

D. No Anticipation by Inherency: Different Mechanisms of Action

The mechanism addressed in the claimed invention is distinct from that described in the Zimmermann patent. The claimed invention is directed to decreasing localized inflammatory responses arising from an ischemia/reperfusion injury in a tissue of a patient comprising intravascularly administering to said patient heparinase enzyme in an effective amount sufficient to decrease neutrophil transmigration through activated endothelium and basement membrane of said vasculature which decreases said localized inflammatory response arising from an ischemia/reperfusion injury. As described above, inflammation requires that leukocytes, such as neutrophils, transmigrate through the activated endothelium and basement membrane. The claimed invention is directed to reducing that transmigration of neutrophils by the administration of heparinase. Therefore, a method of decreasing localized inflammatory response by administering heparinase by the claimed mechanism of action is not described or suggested in the Zimmermann patent.

In the event that the Examiner contends that the Zimmermann patent anticipates the claimed invention by inherency, i.e., that the description of intravascularly administering

heparinase will necessarily result in a decrease localized inflammatory response by reducing neutrophil transmigration across the endothelium and basement membrane, Appellants respectfully submit that such argument is without merit. In the recent case, *Elan Pharmaceuticals, Inc. v. Mayo Foundation for Medical Education and Research*, No. 00-1467 (Fed. Cir. August 30, 2002)(attached as Appendix G), the Federal Circuit reversed a holding of invalidity based on the ground of anticipation by inherency.

The Federal Circuit noted that "[w]hen anticipation is based on inherency of limitations not expressly disclosed in the assertedly anticipating reference, it must be shown that the undisclosed information was known to be present in the subject matter of the reference." *Elan Pharmaceuticals, Inc. v. Mayo Foundation*, ___ F.2d ___ (Fed. Cir. 2002), *citing Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1269 (Fed. Cir. 1991). In *Elan*, the court found that the prior patent made no reference to a particular claim element and the party challenging the validity of the patent provided no evidence that that particular claim element was known to persons of ordinary skill in the field of the invention to be present and therefore, inherent. Rather, the Court stated that "[i]nherency cannot be based on the knowledge of the inventor; facts asserted to be inherent in the prior art must be shown by evidence from the prior art." The Court summarized the law of anticipation as described by Judge Learned Hand:

> No doctrine of the patent law is better established than that a prior patent or other publication to be an anticipation must bear within its four corners adequate directions for the practice of the patent invalidated. If the earlier disclosure offers no more than a starting point for further experiments, if its teaching will sometimes succeed and sometimes fail, if its does not inform the art without more how to practice the new invention, it has not correspondingly enriched the store of common knowledge, and it is not an anticipation.

Elan Pharmaceuticals, Inc., __ F.2d at ___, *citing Dewey v. Almy Chemical Co. v. Mimex Co.*, 124 F.2d 986,989 (2d. Cir. 1942).

Similarly, in the instant case, the Zimmermann patent's disclosure of administration of heparinase does not anticipate the claimed method of reducing localized inflammatory response by reducing transmigration of neutrophils into the endothelium and basement membrane. The Examiner has provided no supporting evidence to document that this is necessarily the case. In fact, such an argument has never been presented by the Examiner and the Examiner relies on Appellants' own specification for the suggestion that ischemia may cause a local inflammatory response. The Zimmermann patent only discloses the effect of heparinase on the release of heparin binding growth factors, which facilitate cell proliferation, one phase in the wound healing process. Moreover, as discussed above, Zimmermann describes that heparinase serves to increase the mobility of cells through the extracellular matrix, a step in the process of inflammation. As such, the Examiner has not established that there is anticipation by inherency because the rejection does not address or provide evidence that heparinase necessarily reduces transmigration of neutrophils into the endothelium and basement membrane as recited in the claim.

E. Unsupported Rejection Under 35 U.S.C. §102(f)

The Office Actions indicate that the claims are alternatively rejected under 35 U.S.C. §102(f), but the basis for this alternative rejection is not stated. Appellants submit that the "examiner must presume the applicants are the proper inventors unless there is proof that another made the invention and that applicant derived the invention from the true inventor." MPEP 706.02(g). As addressed above, appellants respectfully submit that Zimmermann does not, in

fact, describe the present invention and, as such, there is no derivation. Indeed, the instant application and the Zimmermann patent are directed to two different inventions. Accordingly, appellants submit that the Office has not met its burden to establish that the rejection under 35 U.S.C. §102(f) is proper and respectfully request that the Board reverse the rejection.

F. Zimmermann Cannot Be Applied Under 35 U.S.C. §103

To the extent that the Zimmermann is not an anticipating reference, Appellants contend that Zimmermann cannot be applied as a reference under 35 U.S.C. §102(e) in an obviousness rejection. The Zimmermann patent was commonly assigned to Ibex Technologies R and D, Inc., and was subsequently assigned together to BioMarin Enzymes, Inc. in the assignment attached as Appendix A. Accordingly, under 35 U.S.C. 103(c), the commonly assigned Zimmermann patent is not appropriately applied in an obviousness rejection.

IX. CONCLUSION

For the reasons advanced above, Appellants request that the Board of Patent Appeals and Interferences reverse the outstanding rejection and objection, remand the application to the Examiner, and direct the Examiner to issue a Notice of Allowance.

Respectfully Submitted,

Maria L. Maebius
Registration No. 42,967

Hale and Dorr, LLP
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Washington, DC 20004
TEL 202.942.8585
FAX 202.942.8484
Date: November 4, 2002

PATENT ASSIGNMENT (UNITED STATES)

WHEREAS "IBEX Technologies R and D, Inc." is the registered applicant and owner of the patents and related patent applications listed on Schedule "A" and Schedule "B" hereto;

WHEREAS "IBEX Technologies R and D, Inc." is a clerical misstatement of the correct corporate name of Technologies IBEX R & D Inc.,

WHEREAS "IBEX Technologies R and D, Inc." and Technologies IBEX R & D Inc. are one and the same entity;

WHEREAS by an agreement entitled "Agreement to Purchase or License the Intellectual Property" made as of and with effect from the 26th day of May, 1993, Technologies IBEX R & D Inc. did convey all of its right, title and interest in and to the patents and related patent applications listed on Schedule "A" and Schedule "B" hereto effective as of the 29th day of December 1995, to Technologies IBEX Inc.;

WHEREAS Technologies IBEX Inc. is the French form of the corporate name of IBEX Technologies Inc., and Technologies IBEX Inc. and IBEX Technologies Inc. are one and the same entity;

WHEREAS IBEX Technologies Inc. has made applications in its own right for patents as set forth on Schedule "A" and Schedule "B" hereto in the names of "IBEX Technologies" and "IBEX Technologies, Inc.";

WHEREAS "IBEX Technologies" and "IBEX Technologies, Inc." are one and the same entity as IBEX Technologies Inc.;

WHEREAS IBEX Technologies Inc. did transfer and convey all of its right, title and interest in and to certain of the patents and related patent applications set forth in Schedule "A" and Schedule "B" hereto to IBEX Technologies LLC, by an agreement entitled "Technology Transfer and Marketing Agreement " made as of and with effect from the 11th day of December, 1996;

WHEREAS IBEX Technologies LLC has made applications in its own right for patents as set forth on Schedule "A" and Schedule "B" hereto;

AND WHEREAS, by an agreement entitled "United States Asset Purchase

Agreement" made as of and with effect from the 9th day of October, 2001, IBEX Technologies Inc., IBEX Pharmaceuticals Inc., IBEX Technologies LLC, IBEX Technologies Corp., and Technologies IBEX R&D Inc. did transfer, assign, convey, set over, and sell, as their interests may appear, to Biomarin Enzymes Inc., certain intellectual property, including the patents and related patent applications set forth on Schedule "A" and Schedule "B" hereto;

In consideration of Ten Dollars ($10.00), and other good and valuable consideration, the receipt of which is hereby acknowledged **IBEX TECHNOLOGIES LLC**, the successor-in-interest to the registered patent owner, IBEX Technologies R and D, Inc, the office address of which is **900 Market Street, Suite 200, Wilmington, Delaware UNITED STATES OF AMERICA 19801, U.S.A.,:**

Does hereby sell, assign and transfer to **BIOMARIN ENZYMES INC.**, having a place of business at **371 Bel Marin Keys Boulevard, Suite 210, Novato, California 94949, U.S.A.**, (the "Assignee") its successors, assigns and legal representatives, the entire right, title and interest for The United States, and all other countries and jurisdictions, in and to any and all inventions and improvements which are disclosed in the patents and patent applications listed in Schedule "A" and Schedule "B" hereto and all divisional, continuation, continuation-in-part, substitute, renewal, reissue, and all other applications for Letters Patent which have been or shall be filed in the United States or elsewhere on any of said inventions and improvements; and in and to all original and reissued patents which have been or shall be issued in the United States or elsewhere on said inventions and improvements;

Does hereby agree that said Assignee may apply for and receive Letters Patent and re-issue patents for said inventions and improvements in its own name; and that, when requested, without charge to but at the expense of said Assignee, its successors, assigns and legal representatives, to carry out in good faith the intent and purpose of this assignment, the undersigned will execute all divisional, continuation, continuation-in-part, substitute, renewal, reissue, and all other patent applications on any and all said improvements; execute all rightful oaths, assignments, powers of attorney and other papers; communicate to said Assignee, its successors, assigns, and representatives, all facts known to the undersigned relating to said

improvements and the history thereof; and generally do everything possible which said Assignee, its successors, assigns or representatives shall consider desirable for aiding in securing and maintaining proper patent protection for said improvements and for vesting title to said improvements and all applications for patents and all patents on said improvements, in said Assignee, its successors, assigns and legal representatives;

Does hereby authorize and request the Commissioner of Patents of the United States and of all foreign countries to issue any Letters Patent granted for any invention or improvement disclosed in any patent applications listed in Schedule "A" and Schedule "B" or on any subsequently filed divisional, continuation, continuation-in-part, substitute, renewal, reissue and all other applications for Letters Patent which have been or shall be filed in The United States or elsewhere on any of said inventions or improvements, to Assignee, its successors, assigns and legal representatives, as the assignee of the entire interest in and to said inventions or improvements; and

Does hereby covenant with said Assignee, its successors, assigns and legal representatives that no assignment, grant, mortgage, license or other agreement affecting the rights and property herein conveyed has been made to others by the undersigned, and that full right to convey the same as herein expressed is possessed by the undersigned.

The parties have requested that this Agreement and all communications and documents relating hereto be expressed in the English language. Les parties ont exige que ce contrat ainsi que tous documents s'y rattachant soient rediges dans la langue anglaise.

Executed at Montreal, Quebec this 31st day of October, 2001.

IBEX TECHNOLOGIES LLC

Per: _____

I have authority to bind the corporation.

NOTARIAL ACKNOWLEDGEMENT
PROVINCE OF QUEBEC

PROVINCE OF QUEBEC)
COUNTY OF MONTREAL)

On October 31, 2001 before me, the undersigned, a Notary or Commissioner of Oaths in and for the Province of Quebec, County of Montreal, personally appeared Paul Bachr known to me or proved to me on the basis of satisfactory evidence to be an authorized officer of IBEX that he/she executed the within instrument, and further acknowledged to me that such corporation executed the within instrument pursuant to its bylaws or a resolution of Board of Directors.

WITNESS my hand and official seal this 31st day of Oct., 2001.

Claire Bergeron
NOTARY or COMMISSIONER OF OATHS

CLAIRE BERGERON 68,592

SCHEDULE A: U.S. PATENTS AND PATENT APPLICATIONS

Patent Title	Patent No./Application No.	Registered Assignee
Attenuation of Wound Healing Processes	5,997,863	IBEX Technologies R and D, Inc.
Use of Heparinases to Decrease Inflammatory Responses	US Application No. 60/004,622	IBEX Technologies R and D, Inc.
Nucleic Acid Sequences and Expression Systems for Heparinase II and Heparinas III Derived from Flavobacterium Heparinum	5,681,733	IBEX Technologies
(Method for) Enzymatic Neutralization of Heparin	5,262,325	IBEX Technologies, Inc.
Heparinase Free of an Anticoagulant Component from Flavobacterium Heparinum	5,338,677	IBEX Technologies, Inc.
Flavobacterium Heparinum Expression Syste	USSN 09/768,873	IBEX Technologies LLC

SCHEDULE B: WORLDWIDE PATENTS AND PATENT APPLICATIONS

Patent Title	Patent No./Application No.	Registered Assignee
Attenuation of Wound Healing Processes	707007 Australia 95926646.3 European 8-504443 Japan WO9601648	IBEX Technologies R and D, Inc.
(Method for) Enzymatic Neutralization of Heparin	658418 Australia E186.217 Austria 537325 Belgium 537325 Denmark 537325 France 69230243.3 Germany 537325 Greece 537325 Italy 2,542,780 Japan 537325 Luxembourg 537325 Monaco 537325 Netherlands 537325 Spain 537325	IBEX Technologies, Inc.

	Sweden 537325 Switzerland 537325 United Kingdom WO9217203	
Heparinase Free of an Anticoagulant Component from Flavobacterium Heparinum	EPO537325	IBEX Technologies, Inc.
Flavobacterium Heparinum Expression System	PCT 104385.132	IBEX Technologies LLC

APPENDIX B

1. A method to decrease localized inflammatory responses arising from an ischemia/reperfusion injury in a tissue of a patient comprising intravascularly administering to said patient heparinase enzyme in an effective amount sufficient to decrease neutrophil transmigration through activated endothelium and basement membrane of said vasculature which decreases said localized inflammatory response arising from an ischemia/reperfusion injury.

2. The method of claim 1, wherein said administration of said heparinase enzyme removes and digests heparin and heparan sulfate from endothelial cell surfaces and extracellular matrices of said tissue.

3. The method of claim 1, wherein said administration of said heparinase enzyme decreases the accumulation of leukocytes in tissue adjacent to endothelial cell surfaces and extracellular matrices.

4. The method of claim 1, wherein said administration of said heparinase enzyme inhibits leukocyte extravasation by releasing immobilized chemokines from the endothelium.

5. The method of claim 1, wherein said administration of said heparinase enzyme inhibits leukocyte rolling on endothelium.

6. The method of claim 1, wherein said heparinase enzyme is expressed from a recombinant nucleotide sequence, in *Escherichia coli* or *Flavobacterium heparinum.*

7. The method of claim 1, wherein said heparinase enzyme is expressed from a recombinant nucleotide sequence in an organism in which it does not naturally occur.

18. The method of claim 1, wherein said heparinase enzyme is heparinase III.

19. The method of claim 1, wherein said ischemia/reperfusion injury is selected from the group consisting of myocardial infarction, stroke, organ transplant, traumatic shock, cardiovascular surgery.

2 entries found for **ischemia**.

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is·che·mi·a Pronunciation Key (ĭ-skē'mē-ə)
n.

A decrease in the blood supply to a bodily organ, tissue, or part caused by constriction or obstruction of the blood vessels.

[New Latin ischaemia, from Greek iskhaimos, *a stopping of the blood* : iskhein, *to keep back*; see segh- in Indo-European Roots + haima, *blood*.]

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i·sche'mic *adj.*

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ischemia

ischemia: in CancerWEB's On-line Medical Dictionary

Try your search for "ischemia" at:

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- Overture - Search the Web
- Roget's Thesaurus - Search for synonyms and antonyms

Dictionary.com Look up: reperfusion OK
Search: Dictionary Thesaurus

2 entries found for **reperfusion.**

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re·per·fu·sion Pronunciation Key (rē'pər-fyo͞o'zhən)
n.

The restoration of blood flow to an organ or tissue that has had its blood supply cut off, as after a heart attack.

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reperfusion

reperfusion: in CancerWEB's On-line Medical Dictionary

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Review

The inflammatory response in myocardial infarction

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Abstract

One of the major therapeutic goals of modern cardiology is to design strategies aimed at minimizing myocardial necrosis and optimizing cardiac repair following myocardial infarction. However, a sound understanding of the biology is necessary before a specific intervention is pursued on a therapeutic basis. This review summarizes our current understanding of the cellular and molecular mechanisms regulating the inflammatory response following myocardial ischemia and reperfusion. Myocardial necrosis induces complement activation and free radical generation, triggering a cytokine cascade initiated by Tumor Necrosis Factor (TNF)-α release. If reperfusion of the infarcted area is initiated, it is attended by an intense inflammatory reaction. Interleukin (IL)-8 synthesis and C5a activation have a crucial role in recruiting neutrophils in the ischemic and reperfused myocardium. Neutrophil infiltration is regulated through a complex sequence of molecular steps involving the selectins and the integrins, which mediate leukocyte rolling and adhesion to the endothelium. Marginated neutrophils exert potent cytotoxic effects through the release of proteolytic enzymes and the adhesion with Intercellular Adhesion Molecule (ICAM)-1 expressing cardiomyocytes. Despite this potential injury, substantial evidence suggests that reperfusion enhances cardiac repair improving patient survival: this effect may be in part related to the inflammatory response. Monocyte Chemoattractant Protein (MCP)-1 is also markedly upregulated in the infarcted myocardium inducing recruitment of mononuclear cells in the injured areas. Monocyte-derived macrophages and mast cells may produce cytokines and growth factors necessary for fibroblast proliferation and neovascularization, leading to effective repair and scar formation. At this stage expression of inhibitory cytokines such as IL-10 may have a role in suppressing the acute inflammatory response and in regulating extracellular matrix metabolism. Fibroblasts in the healing scar undergo phenotypic changes expressing smooth muscle cell markers. Our previous review in this journal focused almost exclusively on reduction of the inflammatory injury. The current update is prompted by the potential therapeutic opportunity that the open vessel offers. By promoting more effective tissue repair, it may be possible to reduce the deleterious remodeling, that is the leading cause of heart failure and death. Elucidating the complex interactions and regulatory mechanisms responsible for cardiac repair may allow us to design effective inflammation-related interventions for the treatment of myocardial infarction. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cytokines; Infarction; Infection/inflammation; Ischemia; Reperfusion

Abbreviations: α-SMAc, α-Smooth Muscle Actin; bFGF, basic Fibroblast Growth Factor; CSIF, Cytokine Synthesis Inhibitory Factor; DCFH, dichlorofluorescein; ICAM-1, Intercellular Adhesion Molecule-1; IL, interleukin; IP-10, Interferon γ-Inducible Protein-10; LFA-1, Leukocyte Function Antigen-1; LPS, lipopolysaccharide; LTB4, Leukotriene B4; LTC4, Leukotriene C4; MCP-1, Monocyte Chemoattractant Protein-1; M-CSF, Macrophage Colony-Stimulating Factor; MMP, matrix metalloproteinase; NADP, nicotinamide-adenine dinucleotide phosphate; NF-κB, Nuclear Factor-κB; PAF, Platelet Activating Factor; PAF-AH, Platelet Activating Factor-Acetylhydrolase; PDGF, Platelet-Derived Growth Factor; PSGL-1, P-Selectin Glycoprotein Ligand-1; ROS, reactive oxygen species; SCF, Stem Cell Factor; sCR1, Soluble Complement Receptor Type 1; SMemb, embryonic isoform of smooth muscle myosin heavy chain; SOD, superoxide dismutase; TIMP-1, Tissue Inhibitor of Metalloproteinases-1; TGF-β, Transforming Growth Factor-β; TNF-α, Tumor Necrosis Factor-α; TNFR, Tumor Necrosis Factor Receptor; u-PA, Urokinase-type Plasminogen Activator; VEGF, Vascular Endothelial Growth Factor; VLA-5, Very Late Antigen-5

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1. Introduction

Myocardial infarction is associated with an inflammatory reaction, which is a prerequisite for healing and scar formation [1–4]. Coronary artery occlusion critically reduces blood flow to the portion of the myocardium subserved, markedly impairing the energy metabolism. In occlusions of the coronary arteries as short as 5 min, functional abnormalities of the reperfused myocardium are observed for as long as 24–48 h. These abnormalities are not attended by lethal injury and the ischemic myocardium ultimately recovers. This transient functional abnormality ('stunned myocardium') is related to reactive oxygen formation [5,6] but shows little if any evidence of an inflammatory reaction. However, ischemia of significant duration to induce infarction does result in an inflammatory response; this response is both accelerated and augmented if the ischemic tissue is reperfused.

The first experimental evidence that inflammation can extend myocardial injury came as the result of implementing anti-inflammatory strategies in animal models of myocardial ischemia and reperfusion. The systemic administration of corticosteroids was shown to decrease infarct size in a canine model of experimental myocardial infarction [7]. This early evidence led to a clinical study using methylprednisolone in patients with acute myocardial infarction, which resulted in catastrophic results, increasing the incidence of ventricular arrhythmias and extending infarct size [8]. Subsequent investigations suggested that corticosteroids inhibit the inflammatory process decreasing the number of infiltrating leukocytes, but also delay healing and collagen deposition [9]. This observation has been augmented by substantial evidence that reperfusion improves tissue repair and that this effect is mediated by enhancement of the inflammatory response [10–14]. Thus, there is a need for a better understanding of the cellular and molecular events associated with myocardial ischemia and reperfusion in order to develop more site-specific interventions that could mitigate inflammatory injury during early reperfusion without interfering with myocardial healing.

Subsequent experimental studies used various approaches to inhibit the inflammatory response in myocardial infarcts: reducing the generation of chemotactic factors by complement depletion [15], or administration of lipoxygenase inhibitors [16] and leukotriene B_4 (LTB4) antagonists were successful in limiting infarct size. Approaches that reduced neutrophil number such as anti-neutrophil antibodies [17], neutrophil depleting antimetabolites [18] or neutrophil filters [19] were also successful in reducing ischemia-related injury in some models. Finally, free radical scavengers, expected to protect against neutrophil-derived reactive oxygen species were also effective in reducing infarct size or sensitivity to ischemia [20].

This review highlights the mechanisms responsible for the regulation of the inflammatory response following experimental myocardial infarction. This process is dependent on a complex interaction between a variety of pleiotropic inflammatory mediators. Understanding of the basic mechanisms regulating the reaction to injury is crucial for the development of site-specific cell biological strategies of intervention to both reduce injury and promote repair.

2. Humoral inflammatory response

2.1. Initiation of the inflammatory process

2.1.1. Complement activation

Hill and Ward [21] were the first to demonstrate that ischemic myocardial injury can activate the complement cascade in a rat model of myocardial infarction. Subsequently Pinckard and colleagues [22] suggested that myocardial cell necrosis results in the release of subcellular membrane constituents rich in mitochondria, which are capable of triggering the early acting components (C1, C4, C2 and C3) of the complement cascade. Rossen and colleagues [23] have suggested that during myocardial ischemia, mitochondria, extruded through breaks in the sarcolemma, unfold and release membrane fragments rich in cardiolipin and protein. By binding C1 and supplying sites for the assembly of later acting complement components, these subcellular fragments provide the means to disseminate the complement-mediated inflammatory response to ischemic injury. mRNA and proteins for all the components of the classical complement pathway are upregulated in areas of myocardial infarcts [24,25]. Complement activation may have an important role in mediating neutrophil and monocyte recruitment in the injured myocardium. Dreyer and coworkers [26] showed that postischemic cardiac lymph contains leukocyte chemotactic activity, which is maximal during the first hour of reperfusion with washout within the next 3 h. Neutralizing antibodies to C5a added in vitro completely inhibited the chemotactic activity of postischemic cardiac lymph during that period. Other studies demonstrated that monocyte chemotactic activity in cardiac lymph collected in the first hour of reperfusion is wholly attributable to C5a [27].

Blocking activation of the complement system can be achieved by consumptive depletion (such as with cobra venom factor injection), by antibody-induced inhibition of individual complement components (e.g. C5), or by infusion of the soluble form of complement receptor type 1 (sCR1) [28]. Complement depletion using cobra venom factor injection at the time of experimental coronary artery occlusion has been shown to attenuate myocardial necrosis in a variety of animal models [15,29]. However, conclusions derived from these studies with a focus on complement depletion overlook the prior systemic activation that may result in deactivation of neutrophils. Weisman and

coworkers have demonstrated that infusion of soluble human complement receptor type 1 (sCR1) significantly decreased infarct size in a rat model of myocardial ischemia and reperfusion [30]. This study raises the possibility that interference with precisely targeted products of the complement system may reduce myocardial injury [31,32].

2.1.2. Reactive oxygen species

Reactive oxygen species (ROS) are molecules with unpaired electrons in their outer orbit. They have the potential to directly injure cardiac myocytes and vascular cells and may be involved in triggering inflammatory cascades through the induction of cytokines [33,34]. Reactive oxygen species have been shown to exert a direct inhibitory effect on myocardial function in vivo and have a critical role in the pathogenesis of myocardial stunning [5]. In addition, Granger and colleagues [35] have provided evidence for a potential role of reactive oxygen in leukocyte chemotaxis. Potential mechanisms through which reactive oxygen intermediates may generate a leukotactic stimulus include complement activation [36], induction of P-selectin expression [37,38], chemokine upregulation [39,40], and increase in the ability of endothelial ICAM-1 to bind to neutrophils [41].

Most of the evidence implicating ROS in the pathophysiology of myocardial infarction is derived from investigations using free radical scavengers. Jolly and coworkers [20] demonstrated that the combination of the antioxidant enzymes superoxide dismutase and catalase significantly reduced infarct size in dogs undergoing experimental myocardial ischemia and reperfusion. Other investigators found similar beneficial effects of antioxidant interventions in experimental models of myocardial infarction. However, there is a significant number of studies describing a failure of antioxidants to prevent injury or demonstrating an early protective effect, that waned with increased duration of reperfusion [42–44]. Recently, transgenic mice that overexpress copper, zinc superoxide dismutase (SOD1) showed significant protection from postischemic injury [45]. In addition, mice overexpressing MnSOD demonstrated a significant decrease in infarct size in Langendorf-perfused hearts undergoing left coronary artery ligation [46]. Unfortunately, two clinical studies using recombinant human superoxide dismutase in patients with acute myocardial infarction undergoing thrombolysis [47] or balloon angioplasty [48] demonstrated no significant improvement in left ventricular function. Both studies had a small sample size. In addition, prolonged coronary occlusion (>2 h) is usually present in the clinical setting of reperfused myocardial infarction and may cause extensive irreversible myocardial damage, leaving fewer myocytes to be affected by free radical-mediated injury [33,49].

2.1.3. The cytokine cascade

Experimental myocardial infarction is associated with the coordinated activation of a series of cytokine and adhesion molecule genes. A critical element in the regulation of these genes involves the complex formed by NF-κB and Iκβ [50]. NF-κB is activated by a vast number of agents, including cytokines (such as TNF-α and IL-1β) and free radicals. The genes regulated by the NF-κB family of transcription factors are diverse and include those involved in the inflammatory response, cell adhesion and growth control [51]. NF-κB activation has been demonstrated in various models of experimental myocardial ischemia and reperfusion [52–54]. Recently, in vivo transfer of NF-κB decoy oligodeoxynucleotides to bind the transcriptional factor, blocking inflammatory gene activation, reduced the extent of myocardial infarction following reperfusion [55].

The mechanisms responsible for triggering the cytokine cascade in the infarcted myocardium have only recently been investigated. Studies from our laboratory [56–58] indicated a role for preformed mast cell-derived mediators in initiating the cytokine cascade ultimately responsible for ICAM-1 induction in the reperfused canine myocardium. Mast cells have been recognized as an important source of preformed and newly synthesized cytokines, chemokines and growth factors. Gordon and Galli [59,60] identified mouse peritoneal mast cells as an important source of both preformed and immunologically-induced TNF-α. The constitutive presence of TNF-α in canine cardiac mast cells led us to postulate that mast cell-derived TNF-α may be released following myocardial ischemia, representing an 'upstream' cytokine responsible for initiating the inflammatory cascade.

We used a canine model of circumflex coronary occlusion and reperfusion, developed in our laboratory [61] that allows collection of cardiac lymph from chronically instrumented animals, in which inflammatory sequelae of the instrumentation surgery have dissipated. Our experiments demonstrated a rapid release of histamine and TNF-α bioactivity in the early post-ischemic cardiac lymph [56]. In addition, histochemical studies indicated mast cell degranulation in ischemic, but not in control sections of canine myocardium. These findings suggested rapid mast cell degranulation and mediator release following myocardial ischemia. C5a, adenosine and reactive oxygen may represent the stimuli responsible for initiation of mast cell degranulation. Furthermore, in vitro experiments showed that early post-ischemic cardiac lymph is capable of inducing IL-6 expression in canine mononuclear cells. Incubation with a neutralizing antibody to TNF-α in part inhibited IL-6 upregulation suggesting an important role for TNF-α as the upstream cytokine inducer. Mast cell degranulation appears to be confined in the ischemic area and results in rapid release of TNF-α, inducing IL-6 in infiltrating mononuclear cells.

Obviously, the role of TNF-α in myocardial infarction is much more complex than simply serving as a trigger of a cytokine cascade [62,63]. Recent experiments investigated

the role of TNF-α signaling in the infarcted myocardium using mice lacking TNF receptors [64]. TNFR1/TNFR2 double receptor knockout mice undergoing left coronary artery ligation had significantly higher infarct size and increased myocyte apoptosis when compared with wild-type controls [64]. These findings suggested that TNF-α may induce a cytoprotective signal capable of preventing or delaying the development of myocyte apoptosis following myocardial infarction.

Other studies have documented the persistent expression of TNF-α in a model of left anterior descending artery coronary occlusion in the rat [65]. TNF-α expression during the healing phase was not confined to the infarct or peri-infarct zone, but was also localized in the normal non-infarcted myocardium, in which remodeling was ongoing. Sustained TNF-α expression may have a role in the reparative process following myocardial infarction [66].

3. Cell-mediated inflammatory response

3.1. Neutrophil infiltration in reperfused myocardial infarcts

Neutrophil depletion in animals undergoing reperfused myocardial infarction led to a marked decrease in infarct size [17] suggesting that a significant amount of myocardial injury induced by coronary artery occlusion followed by reperfusion may be neutrophil-dependent [67,68]. Neutrophils may release oxidants and proteases and possibly express mediators capable of amplifying cell recruitment [69–71].

One of the earliest sequelae of reperfusion involves neutrophil trapping in the microvasculature. Engler and coworkers [19,72] demonstrated that entrapment of leukocytes in the microcirculation precedes their role in an inflammatory reaction. Neutrophils are large and stiff cells and may adhere to capillary endothelium preventing reperfusion of capillaries following coronary ischemia. The mechanism by which neutrophil trapping occurs in the microvessels is likely to be multifactorial. Chemotactic factors rapidly induce neutrophils to change shape and to become less deformable. Neutrophils also release a variety of autacoids, which induce vasoconstriction and platelet aggregation, such as thromboxane B_2 and LTB_4. Neutrophil interaction with endothelial cells via specific adhesion molecules results in their margination and adhesion to the endothelium. The most dramatic and pathologically significant microvascular abnormality is known as the no reflow phenomenon and has also been directly linked to neutrophil localization. Ambrosio and coworkers [73,74] demonstrated in a canine model that the occurrence of areas of markedly impaired perfusion in postischemic myocardium is related only in part to an inability to reperfuse certain areas on reflow. The delayed, progressive fall in flow to areas that initially received adequate reperfusion appeared to be a more important factor. This phenomenon develops in regions receiving no collateral flow during ischemia and is associated with neutrophil accumulation and capillary plugging during late reperfusion.

While changes in cell shape and deformability, and vasoconstriction are important mechanisms for neutrophil accumulation in the ischemic and reperfused myocardium, the bulk of evidence suggests that the more specific interactions between adhesion molecules are the most critical factors in control of neutrophil-induced pathophysiological changes.

3.2. Neutrophil–endothelial interactions and neutrophil transmigration following myocardial ischemia and reperfusion

A better understanding of the molecular interactions between leukocytes and endothelium has given rise to a consensus model of how leukocyte recruitment into tissues is regulated [75,76]. There is increasing evidence that leukocyte–endothelial interactions are regulated by a cascade of molecular steps that correspond to the morphological changes that accompany adhesion. This adhesion cascade has been divided into sequential steps based on visual assessment of the post-capillary venules during the early stages of acute inflammation. In the absence of inflammation, leukocytes are rarely seen to interact with the vessel wall. After the inflammatory stimulus is applied, leukocytes roll along the post-capillary venules (but not arterioles or small arteries) at velocities distinctly below that of flowing blood. Some rolling cells can be seen to arrest and after a few minutes change shape in apparent response to local chemotactic stimuli. Extravasation into the extravascular tissue follows (Fig. 1). Each of these steps requires either upregulation or activation of distinct sets of adhesion molecules (Fig. 2).

3.2.1. Neutrophil rolling: the role of the selectins

The selectin family of adhesion molecules mediates the initial capture of leukocytes from the rapidly flowing bloodstream to the blood vessel, before their firm adhesion and diapedesis at sites of tissue injury and inflammation [77–79]. The selectin family consists of three closely related cell-surface molecules: L-selectin (CD62L), E-selectin (CD62E), and P-selectin (GMP-140, CD62P). The individual members of the selectin group were designated by prefixes, which were chosen according to the cell type where the molecule was first identified. L-selectin expression is limited to hematopoietic cells, with most classes of leukocytes constitutively expressing L-selectin at some stage of differentiation. The majority of circulating neutrophils, monocytes, eosinophils, T cells and B cells express L-selectin, which is rapidly shed from the surface of these cells following their activation. The broad expression of L-selectin allows it to play a role in the trafficking of all

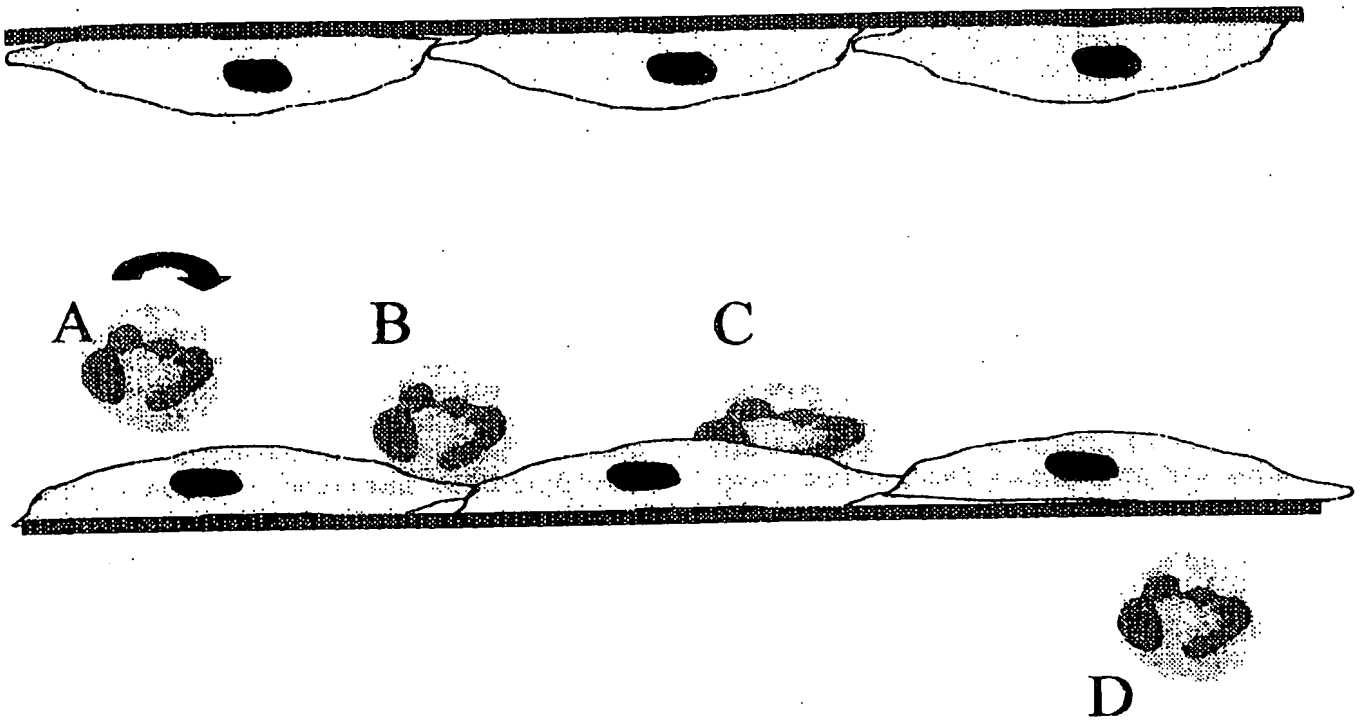

Fig. 1. Diagram illustrating the sequential steps of the adhesion cascade regulating neutrophil localization in post-capillary venules during the early stages of acute inflammation. (A) After the inflammatory stimulus is applied, leukocytes roll along the post-capillary venules at velocities distinctly below that of flowing blood. (B) Some rolling cells can be seen to arrest and after a few minutes change shape (C) in apparent response to local chemotactic stimuli. Extravasation into the extravascular tissue follows (D). Each of these steps requires either upregulation or activation of distinct sets of adhesion molecules.

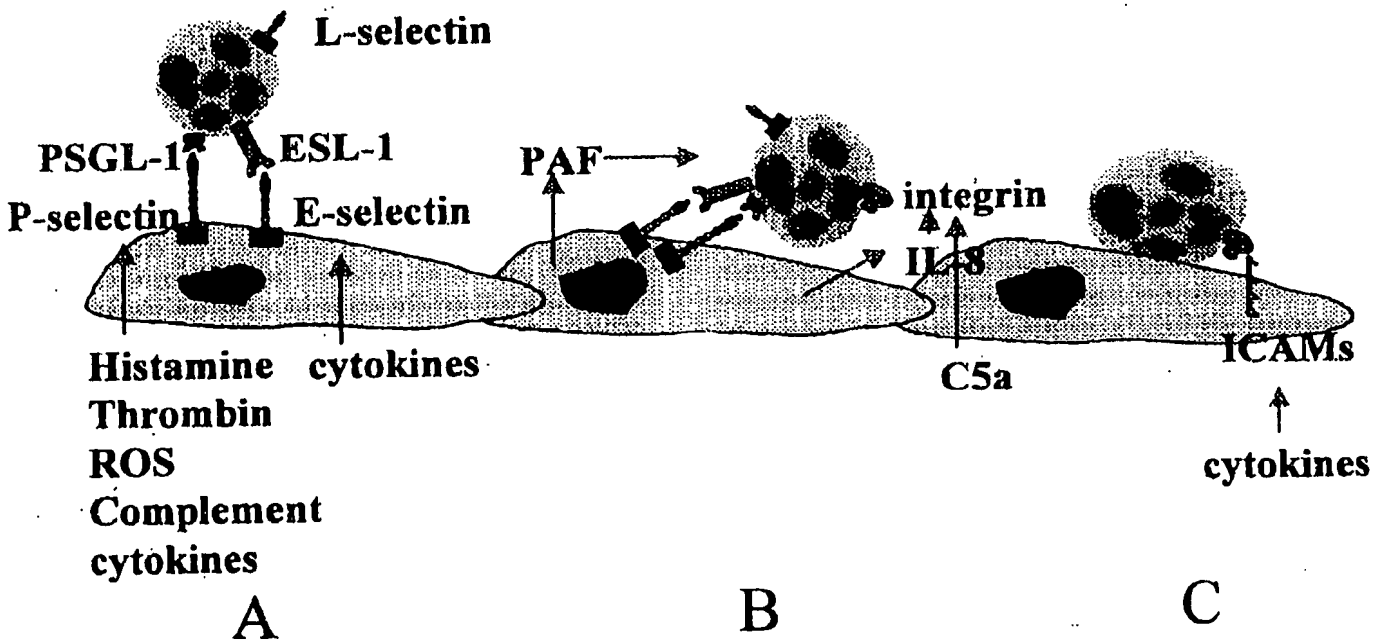

Fig. 2. Endothelial–neutrophil interactions leading to neutrophil transmigration into the injured myocardium. (A) The initial tethering of neutrophils to the endothelial cell surface is mediated by the selectins. (B) This enables the leukocyte to roll along the venular wall and to 'sense' activating factors (such as IL-8 and C5a). These interactions lead to neutrophil integrin activation. (C) Firm adhesion of the leukocyte is mediated through binding of neutrophil integrins to members of the immunoglobulin superfamily expressed in stimulated endothelial cells. Abbreviations: P-Selectin Glycoprotein Ligand-1, PSGL-1; E-Selectin Ligand-1, ESL-1; Reactive oxygen species, ROS; Platelet Activating Factor, PAF; Interleukin-8, IL-8; Intercellular Adhesion Molecule, ICAM.

leukocyte lineages. In contrast, E-selectin is expressed only following de novo synthesis 4–6 h after activation of endothelial cells by cytokines (such as TNF-α, IL-1β) or by bacterial endotoxin [80,81]. P-Selectin is constitutively found in Weibel–Palade bodies of endothelial cells and in α granules of platelets. Within minutes after activation by thrombogenic and inflammatory mediators, P-selectin is mobilized to the cell surface without the need for new protein synthesis. Inducing agents include thrombin, histamine, complement fragments, oxygen-derived free radicals, LTC4/D4 and cytokines. In addition to its regulation through transport to the cell surface, P-selectin is also inducible by cytokines and endotoxin both in vitro [82] and in vivo [83].

One important property of the selectins is that they promote leukocyte attachment and rolling at shear stresses characteristic of post-capillary venules. All three selectins are involved in leukocyte entry into tissues [84–86]. Recently, studies using transgenic mice have improved our understanding of the role of selectins in leukocyte trafficking. L-Selectin-deficient mice have shown a significant reduction in lymphocyte homing to peripheral lymph nodes and leukocyte infiltration to sites of inflammation [87,88]. P-Selectin-deficient mice demonstrated virtually total absence of rolling in mesenteric venules and delayed neutrophil recruitment to the peritoneal cavity upon experimentally-induced inflammation [89]. In contrast to P- and L-selectin mutants, E-selectin-deficient mice have no obvious abnormalities of the inflammatory response [90]. However, P-selectin blocking by treatment of the E-selectin-deficient animals with an anti-murine P-selectin antibody significantly inhibited neutrophil emigration in two distinct models of inflammation [90]. These findings suggest that P- and E-selectin may share overlapping functions [78].

The role of selectins in ischemia and reperfusion is not well defined at present and represents an area of active investigation. L-Selectin is constitutively expressed in neutrophils in a highly specific distribution and its shedding upon activation may be important for leukocyte recruitment [91,92]. P-Selectin surface expression occurs rapidly on endothelial cells under circumstances likely to be seen during ischemia and reperfusion. It is stored in the Weibel–Palade bodies and is rapidly translocated to the endothelial surface in response to thrombin and/or oxidative stress, both of which would be likely to be found upon reperfusion and initiated by thrombolytic agents, and to histamine, which is rapidly released in the ischemic and reperfused myocardium by degranulating mast cells. Experimental studies have suggested that monoclonal antibodies against L-selectin [93] and P-selectin [94] were effective in reducing myocardial necrosis, preserving coronary endothelial function and attenuating neutrophil accumulation in ischemic myocardial tissue in a feline model of ischemia/reperfusion.

Unlike most other cell adhesion molecules that bind to their ligands on the basis of protein–protein interactions, the ligands of the selectins are composed of a scaffold protein, or a lipid carrier molecule, which is modified by certain carbohydrates [95]. Numerous reports have documented that glycolipids bind specifically to the selectins. Sialyl Lewis^x-carrying glycolipids and sialyl Lewis^a-carrying neoglycolipids were shown to support rolling of E-selectin-transfected cells and of L-selectin-expressing leukocytes [96]. P-Selectin glycoprotein ligand-1 (PSGL-1) is the best-characterized selectin ligand to date, fulfilling all criteria for a physiologically relevant ligand [97]. It is the major ligand for P-selectin on human neutrophils as the monoclonal antibody PL-1 to PSGL-1 completely inhibits neutrophil rolling on P-selectin [98]. Recently, peptide analogs of PSGL-1 have been constructed. A soluble form of PSGL-1 has been found to be protective in experimental renal ischemia and reperfusion in rats decreasing renal necrosis due to neutrophil infiltration [99]. Furthermore, a recombinant analog of sPSGL-1 significantly reduced myocardial necrosis in a feline model of coronary occlusion and reperfusion [100].

Recent experiments suggested that P-selectin-deficient mice show decreased infarct size after 30 min of coronary occlusion and 2 h of reperfusion [101]. In contrast, no difference in infarct size was noted after a 60-min ischemic period [101]. In addition, mice with a combined P-selectin and ICAM-1 deficiency demonstrated impaired neutrophil trafficking without a difference in infarct size due to myocardial ischemia and reperfusion [102].

Thus, current concepts suggest a role for selectins in supporting margination under shear stress following experimental myocardial ischemia and reperfusion. The transient nature of this adhesive interaction is important since it allows leukocytes to 'sample' the local endothelium for the presence of specific trigger factors that can activate leukocyte integrins and allow the cascade to proceed.

3.2.2. CD18 and the leukocyte beta 2 integrins

Although rolling appears to be a prerequisite for eventual firm adherence to blood vessels under conditions of flow, selectin-dependent adhesion of leukocytes does not lead to firm adhesion and transmigration unless another set of adhesion molecules, the integrins, is engaged. Integrins are a family of heterodimeric membrane glycoproteins that consist of an α and a β subunit; these subunits are associated through noncovalent bonds and transported to the cell surface as a complex [103]. For neutrophils, firm adhesion requires activation of the β2 (CD18) integrins, which share the beta chain CD18 paired with CD11a (LFA-1), CD11b (Mac-1), or CD11c (p150,95). This results in binding to one of the intercellular adhesion molecules on the surfaces of endothelial cells. LFA-1, Mac-1 and p150,95 have different and yet overlapping roles in adhesion, in part due to their characteristics of expression on leukocytes.

cardiac myocytes. Intercellular adhesion occurred only if the myocytes were stimulated with cytokines inducing ICAM-1 expression and when the neutrophils were stimulated to show Mac-1 activation. In vitro, myocyte ICAM-1 induction could be effected by the cytokines IL-1, TNF-α and IL-6; neutrophil activation could be effected by zymosan-activated serum (a source of C5a) PAF and IL-8. The binding of neutrophils to activated cardiac myocytes was found to be specific for Mac-1–ICAM-1 interaction, and was completely blocked by antibodies to ICAM-1, CD11b and CD18. This interaction was unaffected by antibodies to CD11a, which are capable of blocking neutrophil adhesion to an endothelial cell monolayer. Adhering neutrophils were apparently cytotoxic, as indicated by the sustained contraction often observed in myocytes after neutrophil adhesion.

In other experiments, the mechanisms of neutrophil-induced cytotoxicity were studied [126]. Either neutrophils or cardiac myocytes were loaded with 2′,7′-dichlorofluorescein (DCFH), and the adherence-dependent oxidation of this marker to DCF was monitored under fluorescence microscopy. Using zymosan-activated serum to activate the neutrophils in the presence of cytokine-stimulated cardiac myocytes, neutrophil–myocyte adhesion ensued as described above. When neutrophils were loaded with DCFH, fluorescence appeared almost immediately upon adhesion of the neutrophil to a myocyte suggesting a rapid adhesion-dependent activation of the NADP oxidase system of the neutrophil. In contrast, fluorescence of the cardiac myocytes appeared after several minutes and was rapidly followed by irreversible myocyte contracture. The iron chelator desferrioxamine and the hydroxyl radical scavenger, dimethylthiourea, did not inhibit neutrophil adherence, but completely inhibited the fluorescence and contracture seen in the cardiac myocyte, preventing the neutrophil-mediated injury. In contrast, extracellular oxygen radical scavengers such as superoxide dismutase and catalase or extracellular iron chelators such as starch-immobilized desferrioxamine did not inhibit fluorescence, adhesion or cytotoxicity. Under these experimental conditions, no superoxide production could be detected in the extracellular medium during the neutrophil–myocyte adhesion. These data suggest that Mac-1/ICAM-1 adherence activates the neutrophil respiratory burst resulting in a highly compartmented iron-dependent myocyte oxidative injury.

4.2. Inflammatory myocardial injury in vivo — Possible role of ICAM-1

The pertinence of the in vitro neutrophil-mediated myocyte injury to ischemia/reperfusion injury was suggested by experiments with postischemic cardiac lymph which demonstrate the appearance of C5a activity present during the first 4 h of reperfusion along with neutrophils showing upregulation of Mac-1 on their surface. Postischemic cardiac lymph also contained cytokine activity that upregulated ICAM-1 in isolated cardiac myocytes; this latter activity was neutralized by antibodies to human IL-6 [127]. Further studies were designed to directly evaluate the role of ICAM-1 in myocardial inflammation associated with ischemia and reperfusion.

Using a canine model of reperfused myocardial infarction, Kukielka and coworkers [128] demonstrated ICAM-1 mRNA expression in ischemic myocardial segments as early as 1 h after reperfusion, with marked elevations after longer time intervals. No detectable ICAM-1 mRNA was found in segments with normal blood flow while in the previously ischemic areas. ICAM-1 mRNA appeared as an inverse function of coronary blood flow. At later time points such as 24 h, however, mRNA was found in all myocardial samples (although tissue expression of protein remains confined to the viable border zone), suggesting that circulating cytokines are inducing ICAM-1 mRNA in normal as well as in ischemic areas. The actual expression of ICAM-1 protein was not seen until 3–6 h and was almost exclusively seen in the ischemic area at all time points, implying the possibility of a posttranscriptional regulation of ICAM-1 expression in cardiac myocytes, or, more likely, proteolytic solubilization of surface ICAM-1 on normal cells that may be defective in the jeopardized zone allowing the presence of surface ICAM-1.

Using in situ hybridization techniques, substantial message for ICAM-1 was detected in much of the reperfused viable myocardium by 1 h of reperfusion, adjacent to areas of contraction band necrosis [129]. At 3 h, ICAM-1 mRNA expression occurred in cells in the jeopardized area that appeared viable histologically. In contrast, under circumstances where reperfusion did not occur, ischemic segments did not express ICAM-1 mRNA or ICAM-1 protein in areas of occlusion for periods up to 24 h. It is important to point out that the layers of myocardial cells directly adjacent to the endocardium are spared injury, conserve glycogen and do not express ICAM-1 mRNA in early reperfusion, probably as a result of diffusion across the endocardium from the left ventricular chamber. In addition, this area of induction of ICAM-1 mRNA on the viable border zone region of the infarct is the area where the most intense neutrophil margination and infiltration occur.

Based on these observations, it is reasonable to propose that ICAM-1 facilitates both the emigration of neutrophils in reperfused myocardium and their adherence-dependent cytotoxic behavior. Constitutive levels of ICAM-1 on endothelial cells may be sufficient to support CD18-dependent adhesion and subsequent transendothelial migration in response to chemotactic stimuli, whereas newly expressed ICAM-1 may participate in the myocardial injury associated with reperfusion only under circumstances where a leukotactic gradient and neutrophil activation are present.

4.3. Mechanism of ICAM-1 induction

Because of the capacity of IL-6, present in postischemic

cardiac lymph, to induce myocyte ICAM-1 expression, the expression of IL-6 mRNA in the ischemic and reperfused myocardium was investigated. In these experiments it was demonstrated that IL-6 was rapidly expressed in the same ischemic segments in which ICAM-1 mRNA was found with a peak preceding that of ICAM-1 mRNA [130]. As with ICAM-1 the expression of IL-6 mRNA appeared to be dependent upon reperfusion.

These observations are consistent with the hypothesis that reperfusion initiates a cascade of cytokine-related events leading to IL-6 expression and subsequent induction of ICAM-1 mRNA in the ischemic and reperfused myocardium. It appears that IL-6 synthesis is rapidly induced in cells found within the ischemic and reperfused areas. Mononuclear cells and myocytes in the border zone of myocardial infarcts exhibit reperfusion-dependent expression of IL-6 mRNA within 1 h after reperfusion [131]. TNF-α of mast cell origin may be a crucial factor in upregulating IL-6 in infiltrating cells and initiating the cytokine cascade responsible for myocyte ICAM-1 induction and subsequent neutrophil-induced injury [56].

In addition, IL-6 effects may extend beyond the induction of ligand-specific adhesion of neutrophils to cardiac myocytes. Finkel and coworkers [132,133], have demonstrated that IL-6 may act as a nitric oxide-dependent cardiac depressant and may be associated with stunned myocardium. Furthermore, recent experiments indicated that IL-6 knockout mice demonstrate significantly delayed cutaneous wound healing suggesting a significant role for IL-6 in tissue repair [134].

5. The role of the inflammatory response in the healing myocardial infarction

The potential dangers of anti-inflammatory strategies described above have prompted extensive studies on the role of inflammation in cardiac repair. Both experimental and clinical evidence demonstrate that an open infarct vessel promotes repair even when reperfusion occurs when no myocardial tissue can be salvaged [11,12,135]. The role of reperfusion-induced inflammation in the repair process has been suggested in several experimental models [10,136]. Infiltrating mononuclear cells and mast cells appear to orchestrate the cardiac repair process through a complex cascade involving cytokines and growth factors (Fig. 3). The remainder of this review will focus on this aspect of the inflammatory response.

5.1. Mononuclear cell infiltration

Mononuclear cells infiltrate the infarcted myocardium in the first few hours of reperfusion. The mechanisms responsible for monocyte recruitment have recently been elucidated. Monocyte chemotactic activity in the first hour after reperfusion was wholly attributable to C5a [27]. Transforming growth factor (TGF)-β1 contributed significantly to this chemotactic activity after 60–180 min, and after 180 min, monocyte chemotactic activity in lymph was largely dependent on monocyte chemoattractant protein (MCP)-1 acting in concert with TGF-β1. MCP-1 was rapidly upregulated in the venular endothelium of ischemic myocardial segments. In the absence of reperfusion, no significant MCP-1 induction was noted [137]. Increased monocyte recruitment may lead to more effective healing, explaining the beneficial effects of late reperfusion, when no myocardial tissue can be salvaged.

A recent investigation examined the monocyte–tissue matrix interactions responsible for monocyte accumulation in the infarcted areas demonstrating that reperfusion of ischemic myocardium released diverse fibronectin fragments into cardiac extracellular fluids [138]. Cell-binding fibronectin fragments released under these circumstances induced the proteolysis of monocyte cell-surface Very Late Antigen (VLA)-5. This process appeared to be mediated by serine proteases activated in the course of the response to myocardial injury.

After recruitment in the infarcted territory monocytes differentiate into macrophages. Local upregulation of Macrophage Colony-Stimulating Factor (M-CSF) may have an important role in this process providing the milieu necessary for monocyte maturation [2]. The exact role of the macrophages in the healing scar has not been fully investigated, however they may serve as an important source of cytokines and growth factors [139]. In addition, they may regulate extracellular matrix metabolism through the synthesis of matrix metalloproteinases and their inhibitors [14,140].

5.2. IL-10 as a modulator of the inflammatory response

The inflammatory response ultimately leads to healing and repair of the injured territory. Thus, the molecular signals induced following myocardial infarction may mediate suppression of tissue injury and regulate scar formation. Interleukin-10 (IL-10), a cytokine initially described as cytokine synthesis inhibitory factor (CSIF) is primarily a product of activated Th2 cells and endotoxin-stimulated monocytes [141]. Among the different cell types affected by IL-10, monocyte-macrophages appear to be particularly modified in regard to their function, morphology and phenotype. IL-10 inhibits the production of IL-1α, IL-1β, TNF-α, IL-6 and IL-8 by LPS-activated monocytes, suppressing the inflammatory response. IL-10 also suppresses expression of IL-12, a cytokine primarily produced by activated monocytes and a dominant factor in directing Th1 type responses [141]. Furthermore IL-10 may have a significant role in extracellular matrix formation by modulating expression of metalloproteinases and their inhibitors [142]. The potential role of IL-10 in experimental myocardial infarction has recently been investigated [14,143]. IL-10 mRNA and protein upregulation was demonstrated in the reperfused infarcted myocardium using a canine

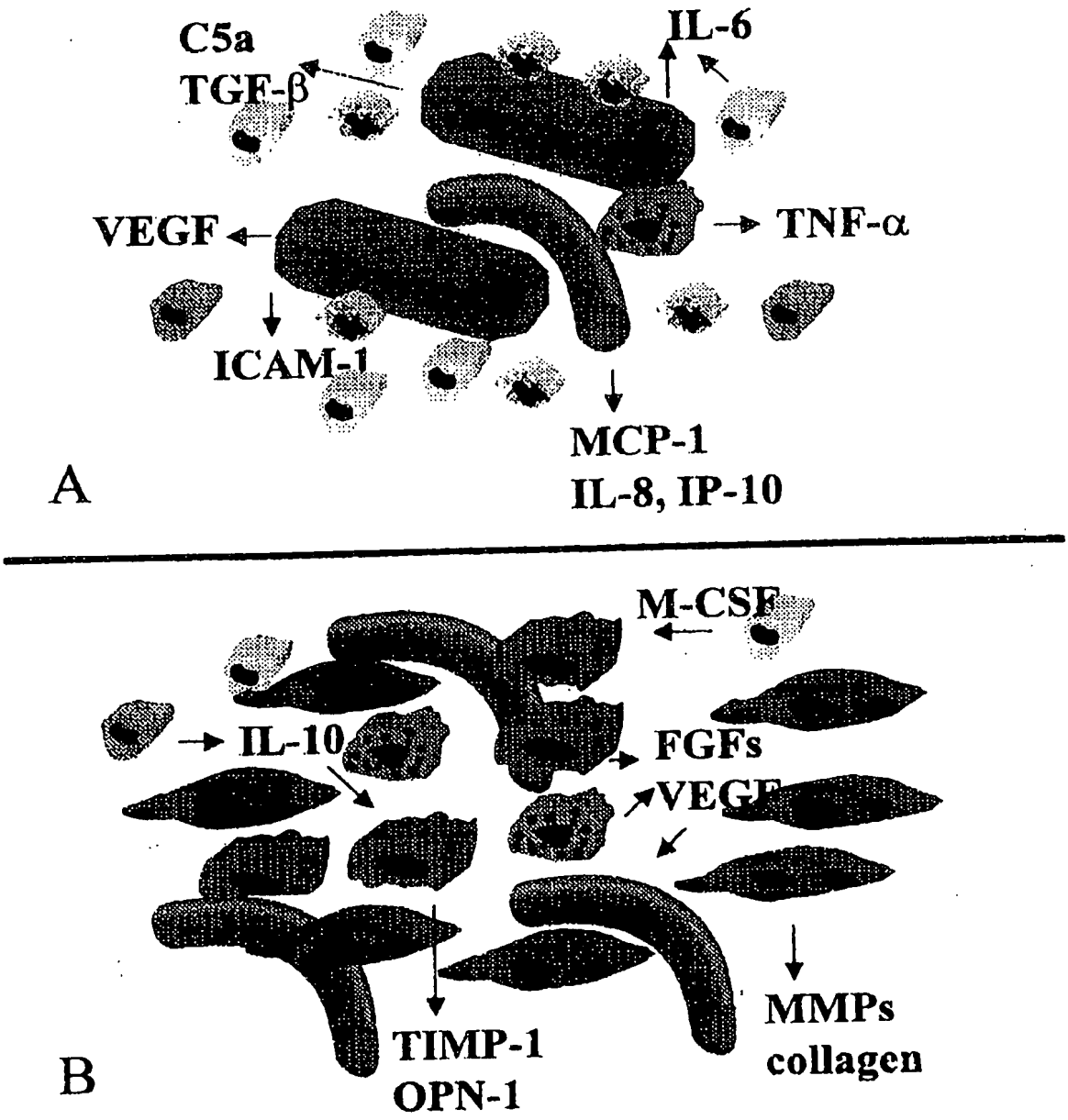

Fig. 3. Schematic diagram illustrating the cellular events associated with the inflammatory response in reperfused myocardial infarcts. (A) In the first 24 h of reperfusion the injured myocardium is infiltrated by neutrophils (gray), monocytes (yellow) and lymphocytes (cyan). Leukocyte recruitment is regulated by complement activation, release of bioactive TGF-β, and induction of chemokines (such as MCP-1 and IL-8). Resident mast cells (green) release preformed mediators (such as histamine and TNF-α) initiating the cytokine cascade, which leads to IL-6 synthesis in mononuclear cells and myocytes (brown). Subsequently, cytokine-stimulated myocytes in the ischemic border zone express ICAM-1 and may be susceptible to neutrophil-mediated cytotoxic injury. At this stage, both angiogenic (such as VEGF, IL-8, MCP-1) and angiostatic factors (such as IP-10) are released; thus the angiogenic process may be delayed until the wound is debrided and a fibrin-based provisional matrix is formed. (B) During the healing phase, infiltrating monocytes differentiate into macrophages (orange). The maturation process may be regulated by local synthesis of M-CSF. Macrophages and mast cells accumulate in the healing scar and secrete a variety of growth factors and cytokines, inducing fibroblast proliferation. Lymphocytes and a subset of the macrophages produce the macrophage-modulating cytokine IL-10, which may have a role in suppressing the inflammatory response and in tissue remodeling by regulating expression of metalloproteinases and their inhibitors. Fibroblasts undergo phenotypic changes expressing α-smooth muscle actin and produce collagen and other extracellular matrix components. At this stage, suppression of the angiostatic chemokine IP-10 may lead to active angiogenesis regulated by a variety of factors, such as VEGF, basic FGF and the angiopoetins. Abbreviations: Transforming Growth Factor-β, TGF-β; IL, Interleukin; Intercellular Adhesion Molecule-1, ICAM-1; Monocyte Chemoattractant Protein-1, MCP-1; Interferon-γ Inducible Protein-10, IP-10; Tumor Necrosis Factor-α, TNF-α; Vascular Endothelial Growth Factor, VEGF; Macrophage Colony-Stimulating Factor, M-CSF; Matrix Metalloproteinases, MMPs; Tissue Inhibitor of Metalloproteinases-1, TIMP-1; Osteopontin-1, OPN-1; Fibroblast Growth Factors, FGFs.

model of myocardial infarction. IL-10 expression was first detected at 5 h and peaked following 96–120 h of reperfusion. In contrast, IL-4 and IL-13, also associated with suppression of acute inflammation and macrophage deactivation, were not expressed. In the ischemic canine heart, CD5 positive lymphocytes were the predominant source of IL-10 in the myocardial infarct. In the absence of reperfusion, no significant induction of IL-10 mRNA was noted. In addition, IL-12, a Th1 related cytokine associated with macrophage activation, was not detected in the ischemic myocardium [14]. In vitro experiments demonstrated that late postischemic cardiac lymph induced Tissue Inhibitor of Metalloproteinases (TIMP)-1 mRNA expression in isolated canine mononuclear cells. This effect was inhibited when the incubation contained a neutralizing antibody to IL-10. These findings suggest that lymphocytes infiltrating the ischemic and reperfused myocardium express IL-10 and may have a significant role in healing by modulating mononuclear cell phenotype and inducing TIMP-1 expression. Furthermore, IL-10 was found to have a role in regulation of the angiogenic in human lymphoid malignancies [144] and in a model of ischemia-induced angiogenesis in mice hindlimb [145]. These studies underscore the importance of IL-10 in the healing process.

Additional investigations indicated that IL-10-deficient mice show an enhanced inflammatory response following experimental myocardial infarction, demonstrated by increased neutrophil recruitment, elevated plasma levels of TNF-α and high tissue expression of ICAM-1 [143]. Thus, IL-10 may have a protective role after myocardial ischemia/reperfusion through the suppression of the acute inflammatory process.

5.3. Mast cell accumulation in the healing scar

As described above, mast cell degranulation is an early source of preformed histamine and TNF-α, modulating the inflammatory response. In later stages, there is a likely role for mast cells in the superbly orchestrated interaction of cells, cytokines, growth factors and extracellular matrix proteins mediating myocardial repair. Macrophages and mast cells provide a rich source of cytokines and growth factors necessary to support fibroblast proliferation and neovessel formation. There is significant evidence that mast cells participate in the fibrotic process [146,147]. Recent studies demonstrated that mast cell numbers increase in the healing phase of reperfused canine myocardial infarcts [148]. The increase in mast cell density was first noted after 72 h of reperfusion. Following 5–7 days of reperfusion, mast cell numbers in fibrotic areas, in which myocytes were fully replaced by scar were markedly higher than the numbers from areas of the same section showing intact myocardium. These experiments failed to demonstrate significant numbers of proliferating mast cells in the healing heart. Although the contribution of mast cell proliferation cannot be ruled out, chemotaxis of circulating mast cell precursors in the healing myocardium may be the predominant mechanism responsible for mast cell accumulation in the ischemic myocardium. Mast cells originate from CD34+ hematopoietic stem cells and circulate as immature precursors in the peripheral blood. Rodewald and colleagues [149] identified a cell population in murine fetal blood that fulfills the criteria of progenitor mastocytes. It is defined by the phenotype Thy-1 (lo) c-kit (hi), expresses RNAs encoding mast cell associated proteases, but lacks expression of the high-affinity immunoglobulin E receptor.

The factors responsible for mast cell accumulation in areas of fibrosis remain to be defined. Stem Cell Factor (SCF) is a potent mast cell chemoattractant that stimulates directional motility of both mucosal- and connective tissue type-mast cells [150]. In addition, several angiogenic factors, such as Platelet-Derived Growth factor (PDGF), Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) have been demonstrated to promote murine mast cell chemotaxis in vitro. However, SCF along with the anaphylatoxins C3a and C5a are the only factors shown to induce migration of human mast cells. Subcutaneous administration of recombinant human SCF to baboons produced a marked expansion of the mast cell population, which was reversed when the cytokine was discontinued [66], providing the first direct evidence that a specific factor can regulate mast cell development in vivo. In a canine model of myocardial infarction, SCF mRNA expression was markedly upregulated in ischemic myocardial segments following 1 h of ischemia and 72 h of reperfusion. At the same time point, an increase in mast cell numbers was noted in the healing myocardium. Immunohistochemical studies showed that SCF immunoreactivity in the healing myocardial scar was predominantly localized in a subset of macrophages. In addition to being a mast cell chemoattractant, SCF critically regulates the maturation and survival of mast cells by suppressing mast cell apoptosis, enhancing mast cell maturation and inducing mast cell adhesion to fibronectin. Furthermore, SCF is capable of inducing substantial mast cell histamine release and can promote the functional activation of mast cells in vivo. All these actions may be important in regulating mast cell growth and activity after myocardial ischemia. Recently, Patella and colleagues [151] demonstrated increased mast cell density and stem cell factor expression in patients with idiopathic and ischemic cardiomyopathy, suggesting that sustained mast cell hyperplasia in cardiomyopathic hearts may contribute to collagen accumulation and fibrosis.

The potential role of mast cells in the healing process remains to be elucidated.

Mast cell degranulation products induce fibroblast proliferation. When activated mast cells were cocultured with fibroblasts they were found to increase collagen synthesis and stimulate fibroblast proliferation, indicating a direct involvement of mast cells in the fibrotic process. Many

mast cell-derived mediators may potentially influence fibroblast growth and function. Histamine has been shown to stimulate fibroblast growth and collagen synthesis in vitro. Tryptase, the most abundant of the proteases found in mast cell granules, induces fibroblast proliferation, stimulates fibroblast chemotaxis and upregulates type I collagen production. Furthermore, mast cells are important sources of TGF-β, bFGF and VEGF, factors that can regulate fibroblast growth, modulate extracellular matrix metabolism and stimulate angiogenesis. Finally, mast cells may influence healing and tissue remodeling by expressing gelatinases A and B, which are implicated in extracellular matrix degradation and angiogenesis [152].

5.4. Fibroblasts and extracellular matrix remodeling

Macrophages, mast cells and lymphocytes create an environment rich in inflammatory cells, capable of regulating neovessel formation, fibroblast proliferation and extracellular matrix metabolism, through the production of a variety of cytokines and growth factors. Fibroblasts produce the extracellular matrix constituents needed to support cell ingrowth and newly formed blood vessels carry oxygen and nutrients necessary to sustain cell metabolism. Willems and colleagues [153] have previously identified and characterized the interstitial nonvascular α-smooth muscle actin (α-SMAc) positive cells, which were present in human myocardial scars 4–6 days after an infarction. These cells are phenotypically modulated fibroblasts termed myofibroblasts [154] that develop ultrastructural and phenotypic characteristics of smooth muscle cells. They are the predominant source of collagen mRNA in healing myocardial infarcts. Myofibroblasts transiently appear during granulation tissue formation and become apoptotic when the scar matures. TGF-β appears to have an important role in myofibroblast differentiation during wound healing by regulating α-SMAc expression in these cells [155]. Persistent expression of α-SMAc by fibroblasts has been described for at least 8 weeks after a nonreperfused myocardial infarct in the rat [156,157]. Cardiac myofibroblasts stain positive for vimentin, but do not express smooth muscle myosin, calponin and desmin [158]. Recent experiments have shown that myofibroblasts in the healing scar express a homologue of the Drosophila tissue polarity gene frizzled (fz2), when migrating into the granulation tissue, which may be involved in the spatial control of cardiac wound repair after infarction [159]. In addition, expression of the embryonal isoform of smooth muscle myosin heavy chain (SMemb) has been demonstrated in reperfused canine myocardial infarcts [10]. SMemb expression may reflect the dedifferentiation and phenotypic plasticity of myofibroblasts following cardiac injury, which may facilitate wound repair. Myofibroblasts are undifferentiated cells that may be capable of assuming a variety of different roles, such as extracellular matrix metabolism, neovessel formation and contractile activity [160–162].

The reparative phase of healing involves activation of proteinases, which are critical for cell migration and extracellular matrix remodeling. Recent studies have demonstrated that deficiency of urokinase-type plasminogen activator (uPA) protected mice undergoing left coronary artery ligation against myocardial rupture [163]. However, uPA -/- mice also showed impaired scar formation and infarct neovascularization. Furthermore, plasminogen-deficient mice showed a profound disturbance in healing suggesting a crucial role for the proteolytic system in regulating cardiac repair [164]. Matrix metalloproteinase (MMP) expression is upregulated in the infarcted myocardium [165,166] and may have a prominent role in extracellular matrix remodeling. Administration of MMP inhibitors [167] and targeted deletion of MMP-9 [168] attenuated left ventricular enlargement in murine myocardial infarction.

5.5. Temporal regulation of angiogenesis in the evolving and healing infarct

Formation of new blood vessels is critical for supplying the healing infarcted myocardium with oxygen and nutrients necessary to sustain metabolism. Angiogenesis is dependent on a complex interaction between extracellular matrix, endothelial cells and pericytes in response to an imbalance in the presence of angiogenic compared to angiostatic factors in the local environment [169–173]. Myocardial infarction is associated with an early release of angiogenic factors in the injured areas. Numerous investigations have indicated that VEGF, IL-8 and bFGF, all potent angiogenic agents, are rapidly induced in the ischemic myocardium [119,174,175] and may have a role in enhancing infarct neovascularization. Recently, we hypothesized that expression of angiostatic factors in the early stages of reperfusion may inhibit onset of angiogenesis until the injured myocardium has been cleared from dead cells and debris by infiltrating phagocytes and a fibrin-rich provisional matrix is formed in order to support ingrowth of new blood vessels [176]. Thus, we examined regulation of the angiostatic CXC chemokine Interferon-γ inducible Protein (IP)-10 [177–180]. IP-10 mRNA expression peaked after 1–3 h of reperfusion and was markedly decreased by 10 h of reperfusion. IP-10 mRNA and protein were localized in the venular endothelium of ischemic myocardial segments. By 24 h of reperfusion, neither IP-10 mRNA nor protein were detected. The earliest histological indication of angiogenesis began at 24 h. Isolated canine jugular vein endothelial cells expressed high levels of IP-10 and IL-8 message upon stimulation with TNF-α and endotoxin. TGF-β but not IL-10 decreased TNF-α-mediated IP-10 expression in canine endothelial cells [176]. In contrast, TNF-α-mediated IL-8 induction was not affected by incubation with IL-10 or TGF-β. We suggest that IP-10 downregulation after an early dramatic peak

following myocardial ischemia and reperfusion may allow unopposed VEGF and IL-8-mediated angiogenic activity. TGF-β may have an important indirect role in promoting angiogenesis following experimental myocardial infarction by suppressing expression of IP-10 [176].

6. Anti-inflammatory strategies following myocardial infarction. Are they doomed to fail?

The importance of the inflammatory cascade in myocardial infarction has been recognized and thoroughly investigated for the last 25 years. A vast body of evidence suggested a role for a variety of inflammatory mediators in myocardial infarction. In addition, numerous experimental studies have shown a dramatic reduction in infarct size with the use of specific anti-inflammatory strategies. However, attempts to mitigate inflammatory injury in clinical practice have, in general, been unsuccessful. The catastrophic experience of the methylprednisolone trial emphasized the need for a better understanding of the cellular and molecular events associated with the inflammatory response to achieve effective suppression of injurious processes without interfering with healing and cardiac repair. Recently, the disappointing results of the phase II anti-CD18 trials [181] led to criticism regarding the usefulness of strategies targeting the inflammatory cascade in myocardial infarction. It has been suggested that these failures may represent the inherent risk of using animal models, which may have fundamental differences from the respective human disease process. Although species-specific effects may be significant in some cases, the most important lesson we have learned from studying experimental myocardial infarction is that a sound understanding of the biology is necessary before a specific intervention is pursued on a therapeutic basis. The inflammatory cascade is based on a complex network of molecular steps mediated by molecules with pleiotropic effects, dictated by critical cellular, spatial and temporal variables. Typical properties of cytokines in networks are redundancy, pleiotropy, synergistic activity and antagonistic effects upon each other. Thus, cytokines and other inflammatory mediators, which may appear reasonable therapeutic targets considering their injurious role in the early stages of the inflammatory response may also be necessary as regulators of cardiac repair. For example, TNF-α and IL-6 may have a role in the initial inflammatory injury associated with myocardial ischemia [56,131], however they may also represent important regulators of myocyte apoptosis and cardiac repair [64,134]. We are only beginning to elucidate the role and significance of various cytokines and growth factors in healing of a myocardial infarction. Successful application of inflammation-related interventions in the treatment of myocardial infarction will require a more complete understanding of the specific molecular steps involved in the regulation of ischemic cardiac injury and repair.

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United States Court of Appeals for the Federal Circuit

00-1467

ELAN PHARMACEUTICALS, INC.
and ATHENA NEUROSCIENCES, INC.,

Plaintiffs-Appellants,

v.

MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH,

Defendant-Appellee.

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Appealed from: United States District Court for the Northern District of California

Judge William H. Alsup

United States Court of Appeals for the Federal Circuit

00-1467

ELAN PHARMACEUTICALS, INC.
AND ATHENA NEUROSCIENCES, INC.,

Plaintiff-Appellants,

v.

MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH,

Defendant-Appellee.

DECIDED: August 30, 2002

Before NEWMAN, GAJARSA, and DYK, Circuit Judges.

Opinion for the court filed by Circuit Judge NEWMAN. Dissenting opinion filed by Circuit Judge DYK.

NEWMAN, Circuit Judge.

Elan Pharmaceuticals, Inc. and Athena Neurosciences, Inc. (collectively "Elan") appeal the decision of the United States District Court for the Northern District of California, granting summary judgment in favor of the Mayo Foundation for Medical Education and Research ("Mayo").[1] The district court held that Elan's two patents in

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[1] Elan Pharmaceuticals, Inc. v. Mayo Foundation for Medical Education & Research, 175 F. Supp.2d 1209 (N.D. Cal. 2000).

suit, United States Patent No. 5,612,486 for "Transgenic Animals Harboring APP Allele Having Swedish Mutation" (the '486 patent) and continuation Patent No. 5,850,003 for "Transgenic Rodents Harboring APP Allele Having Swedish Mutation" (the '003 patent), inventors Lisa McConlogue and Jun Zhao, are invalid on the ground of anticipation by United States Patent No. 5,455,169 for "Nucleic Acids for Diagnosing and Modeling Alzheimer's Disease" (the Mullan patent). We reverse the summary judgment, for the legal requirements of anticipation were not met on the facts of record, and remand for further proceedings.

BACKGROUND

Alzheimer's disease is a progressive neurodegenerative disease that primarily afflicts the elderly. Elan's '486 and '003 patents are directed to transgenic animals whose genetic makeup has been altered so that they are susceptible to Alzheimer's disease. The DNA of these animals has been modified to contain a mutated human gene called the "Swedish mutation," for the gene was isolated from the cells of a Swedish family having an unusually high incidence of early-onset Alzheimer's disease.[2]

The brains of people with Alzheimer's disease contain abnormal tangles and deposits of plaques. At the time of these Elan inventions it was known that a principal

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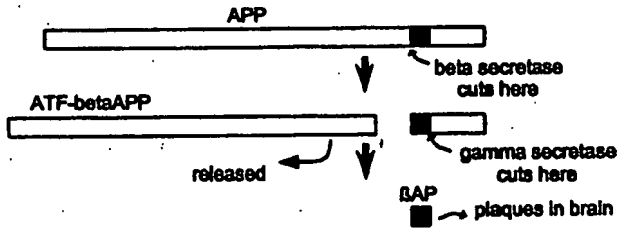
[2] A gene is a segment of DNA. A mutation is a change in a gene and the resulting change in a protein produced by the gene. A gene produces a protein by first copying (transcribing) a portion of DNA into an intermediate strand designated mRNA; the mRNA then produces, through several complex steps, the sequence of amino acids that constitutes the protein. This procedure is called "gene expression." See Bruce Alberts et al., Essential Cell Biology (1998), Ch.6 "DNA," Ch.7 "From DNA to Protein."

component of these plaques is a protein fragment called beta-amyloid peptide (betaAP, also designated βAP and Aβ). The presence of betaAP in the brain is believed to induce or foster formation of the Alzheimer's plaques. It was known that betaAP may be formed when a protein produced in the brain, called the amyloid precursor protein (APP), is cleaved by enzymes in the brain. The Elan patents summarize scientific research in this field, including various reported mutations. Elan explains that an enzyme called beta-secretase cuts the APP molecule between amino acids 596 and 597, releasing a larger protein fragment called the amino terminal fragment (ATF-betaAPP); and an enzyme called gamma-secretase releases the smaller betaAP fragment from the remaining portion of the APP. This mechanism is illustrated in the Elan brief as follows:

Fig.1 - Processing of APP to ßAP and ATF-betaAPP



Humans who do not develop Alzheimer's disease are believed to break down APP in a manner that does not produce significant amounts of betaAP.

The Prior Art

The prior art on which the district court based its summary judgment of anticipation is the Mullan patent. Dr. Mullan had learned of the Swedish family susceptible to Alzheimer's disease, obtained samples of their DNA, isolated the relevant mutated gene, and identified the nature and location of the mutation in the gene as well as in the mutated protein (APP_{sw}) expressed by the gene. Mullan explained that in the Swedish mutation the DNA nucleotides that encode codons 670 and 671[3] replace lysine and methionine, the amino acids normally encoded at these positions, with asparagine and leucine. Mullan states that transgenic animals containing the mutated gene can be used in Alzheimer's disease (AD) research and therapy:

> The invention also provides a transgenic non-human animal containing, in a germ or somatic cell, the mutated nucleic acid of the invention, wherein the animal expresses a human amyloid precursor protein or fragment thereof which encodes an amino acid other than lysine at codon 670 and/or an amino acid other than methionine at codon 671.
>
> * * *
>
> The invention also provides a method of screening for an agent capable of treating AD. The method comprises contacting these transgenic animals or host cell lines with the agent and monitoring the expression, processing or deposition of amyloid precursor protein or fragments thereof.

Mullan, col. 4, lines 36-64. Mullan states that the mutated human gene can be used to create transgenic animals in various ways; for example:

> In yet a further use of the present invention, the mutated gene (i.e., a variant APP codon 670/1 gene) can be excised for use in the creation of transgenic animals containing the mutated gene. For example, an entire human variant APP codon 670/1 allele can be cloned and isolated, either in parts or as a whole, in a suitable cloning vector (e.g., 1Charon35, cosmid, retrovirus or yeast artificial chromosome). The vector is selected

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[3] The mutation positions at codons 670/671 (Mullan) and 596/597 (Elan) are the same, due to differing starting points in the APP chain. See '486 patent, col. 11, lines 29-34.

based on the size of the desired insert and the ability to produce stable chromosome integration.

Col. 11, lines 23-31. Mullan also states that the mutated gene can be transferred to a mouse that preferably will express the variant human APP:

> The human variant APP codon 670/1 gene, either in parts or in whole, can be transferred to a host non-human animal, such as a mouse. As a result of the transfer, the resultant transgenic non-human animal will express one or more variant APP codon 670/1 polypeptides. Preferably, a transgenic non-human animal of the invention will express one or more variant APP codon 670/1 polypeptides in a neuron-specific manner (Wirak et al. (1991) EMBO 10:289). This may be accomplished by transferring substantially the entire human APP gene (encoding a codon 670/1 mutant) including the 4.5 kilobase sequence that is adjacent to and upstream of the first major APP transcriptional start site.

Col. 11, lines 32-43. Mullan discusses the various known procedures of gene transfer, citing scientific articles as to each "approach" used to create transgenic animals:

> One approach to creating transgenic animals is to target a mutation to the desired gene by homologous recombination in an embryonic stem (ES) cell line in vitro followed by microinjection of the modified ES cell line into a host blastocyst and subsequent incubation in a foster mother (see Frohman and Martin, Cell (1989) 56:145). Alternatively, the technique of microinjection of the mutated gene, or a portion thereof, into a one-cell embryo followed by incubation in a foster mother can be used. Certain possibilities for the general use of transgenic animals, particularly transgenic animals that express a wild-type APP fragment, are disclosed in Wirak et al., the EMBO Journal, 10(2) 289-296 (1991); Schilling et al., Gene 98(2) 225-230 (1991); Quon, et al. (1991) Nature 352:239; Wirak, et al. (1991) Science 253:323; and Kawabata, et al. (1991) Nature 354:476. Alternatively, viral vectors, e.g., Adeno-associated virus, can be used to deliver the mutated gene to the stem cell. In addition, such viral vectors can be used to deliver the mutated gene to a developed animal and then used to screen (Mendelson et al. Virology 166:154-165; Wondisford et al. (1988) Molec. Endocrinol. 2:32-39 (1988)).

Col. 11, line 58 to col. 12, line 11. Mullan also states that the mouse gene allele can be mutated to produce a mutation corresponding to the Swedish mutation:

> Site-directed mutagenesis and/or gene conversion can also be used to mutate a murine APP gene allele, either endogenous or transfected, such that the mutated allele does not encode lysine/methionine at the codon

> position in the mouse APP gene that corresponds to codon 670/1 (of APP770) of the human APP gene (such position is readily identified by homology matching of the murine APP gene or APP protein to the human APP gene or APP770 protein). Preferably, such a mutated murine allele would encode asparagine or leucine at the corresponding codon position.

Col. 12, lines 12-21.

It is undisputed that Mullan did not produce a transgenic animal with the Swedish mutation, or determine which of the known procedures would be effective for this purpose, or suggest conditions or details of any method for successful production of the desired animal. Expert witnesses for both sides testified as to the difficulty, uncertainty, unpredictability, and low success rate of each method that has been used to create transgenic animals.

The Elan Patents

The Elan patents describe the production and characteristics of transgenic rodents, specifically mice, whose DNA contains a gene harboring the Swedish mutation, which gene expresses human APP having the Swedish mutation. This APP_{sw} in turn produces human betaAP by action of the mouse enzymes. Expert witnesses for both sides testified as to the unpredictability of the process and the various steps thereof, for not all of the known methods may work, very few attempted gene transfers are successful, and of the relatively few mice that may accept the Swedish gene, not all will express the mutated human APP in a way that is subject to enzymatic cleavage to produce betaAP.

Elan explains that the production of betaAP in the mouse brain is difficult to detect because the betaAP molecule is relatively small. The Elan patents report

detecting the betaAP by detecting the larger cleavage fragment, ATF-betaAPP. Claim 1 of the '486 patent includes this limitation:

> 1. A transgenic rodent comprising
>
> a diploid genome comprising a transgene encoding a heterologous APP polypeptide having the Swedish mutation wherein the amino acid residues at positions corresponding to positions 595 and 596 in human APP695 are asparagine and leucine, respectively,
>
> wherein the transgene is expressed to produce a human APP polypeptide having the Swedish mutation,
>
> and wherein said polypeptide is processed to ATF-betaAPP in a sufficient amount to be detectable in a brain homogenate of said transgenic rodent.

The '003 patent differs from the '486 patent in that the '003 claims include a promoter and a polyadenylation site. Claim 1 of the '003 patent follows:

1. A transgenic rodent comprising

a diploid genome comprising a transgene comprising in operable linkage a promoter, a DNA segment encoding a heterologous APP polypeptide and a polyadenlyation site,

wherein the APP polypeptide has the Swedish mutation whereby the amino acid residues at positions corresponding to positions 595 and 596 in human APP695 are asparagine and leucine, respectively,

wherein the transgene is expressed to produce a human APP polypeptide having the Swedish mutation,

and wherein said polypeptide is processed to ATF-betaAPP in a sufficient amount to be detectable in a brain homogenate of said transgenic rodent.

For both patents, dependent claims 2 and 3 add the limitations that the rodent is murine (mouse) and that the transgene is nonhomologously integrated. These limitations are not asserted to add patentable distinctions. Elan concentrates on the '486 patent on this appeal.

The Mullan patent was prior art based on its filing date, and the examiner granted the Elan patents only after Elan added the final clause to the claims. Elan argues on this appeal that its claims are limited by the presence of detectable ATF-betaAPP in the rodent brain, that this limitation is not shown by Mullan, and thus that as a matter of law the claims cannot be "anticipated." Elan states that "ATF-betaAPP was not even disclosed in humans until after Mullan was filed," and thus that this limitation cannot be deemed "inherent" in the Mullan disclosure.

The district court found that although Mullan does not mention the formation of ATF-betaAPP, its formation is inherent in Mullan's general teachings of transgenic mice with the Swedish mutation. The court found that the Elan claims do not require that the claimed mice be tested for detectable ATF-betaAPP in brain homogenate. Thus the court found that Mullan anticipates the Elan claims, and on summary judgment held the claims of both patents invalid on this ground.

DISCUSSION

The grant of summary judgment on a question of fact requires that "when the facts are viewed in the light most favorable to the non-moving party and all doubts are resolved in favor of the non-movant, there are no genuine issues of material fact and the moving party is entitled to judgment as a matter of law." Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 247-48 (1986). Elan argues that the factual and legal criteria of anticipation were not met. Elan also argues that summary judgment was inappropriate because material facts were in dispute, and that Elan would prevail if the disputed facts were resolved in its favor.

A

To be patented an invention must be new. 35 U.S.C. §§101, 102(a), (e). If it is not new, that is, if it was known to others, it is said to be "anticipated." Hoover Group, Inc. v. Custom Metalcraft, Inc., 66 F.3d 299, 302, 36 USPQ2d 1101, 1103 (Fed. Cir. 1995) ("lack of novelty (often called 'anticipation') requires that the same invention, including each element and limitation of the claims, was known or used by others before it was invented by the patentee"). Anticipation is a question of fact, as is the question of inherency. In re Schreiber, 128 F.3d 1473, 1477, 44 USPQ2d 1429, 1431 (Fed. Cir. 1997). Its proof differs from that for obviousness, 35 U.S.C. §103, in that prior knowledge by others requires that all of the elements and limitations of the claimed subject matter must be expressly or inherently described in a single prior art reference. In re Robertson, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950 (Fed. Cir. 1999); Constant v. Advanced Micro-Devices, Inc., 848 F.2d 1560, 1571, 7 USPQ2d 1057, 1064 (Fed. Cir. 1988). The single reference must describe and enable the claimed invention, including all claim limitations, with sufficient clarity and detail to

establish that the subject matter already existed in the prior art and that its existence was recognized by persons of ordinary skill in the field of the invention. Crown Operations International, Ltd. v. Solutia Inc., 289 F.3d 1367, 1375, 62 USPQ2d 1917, 1921 (Fed. Cir. 2002); In re Spada, 911 F.2d 705, 708, 15 USPQ2d 1655, 1657 (Fed. Cir. 1990) ("the reference must describe the applicant's claimed invention sufficiently to have placed a person of ordinary skill in the field of the invention in possession of it").

The anticipating reference "must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter." PPG Industries, Inc. v. Guardian Industries Corp., 75 F.3d 1558, 1566, 37 USPQ2d 1618, 1624 (Fed. Cir. 1996). When anticipation is based on inherency of limitations not expressly disclosed in the assertedly anticipating reference, it must be shown that the undisclosed information was known to be present in the subject matter of the reference. Continental Can Co. USA, Inc. v. Monsanto Co., 948 F.2d 1264, 1269, 20 USPQ2d 1746, 1749-50 (Fed. Cir. 1991). An inherent limitation is one that is necessarily present; invalidation based on inherency is not established by "probabilities or possibilities." Scaltech, Inc. v. Retec/Tetra, LLC., 178 F.3d 1378, 1384, 51 USPQ2d 1055, 1059 (Fed. Cir. 1999).

B

The district court found that the Elan claims were anticipated by Mullan because use of the standard procedures set forth in Mullan would be expected to produce a statistically small percentage of transgenic mice, and some of these mice would be expected to produce detectable ATF-betaAPP on enzymatic cleavage. The court deemed it irrelevant that the ATF-betaAPP was not described in the prior art. The court found that since the low success rate for gene transfer and expression was known, it

was a matter of statistical probability that a few successful results would be obtained. Thus the district court found that the Elan invention was anticipated by Mullan.

Elan argues that Mullan does no more than teach broad known "recipes" for gene transfer, and that the Mullan disclosure is simply an invitation to experiment, with no assurance of success. That is clearly so. Although Mullan described known procedures for making a transgenic animal, he neither described every element of the claims, nor taught, in terms other than by trial and error and hope, production of a transgenic mouse having detectable ATF-betaAPP in brain homogenate. General instructions to conduct such failure-prone activities as gene transfer between humans and animals, and the ensuing uncertainties with respect to gene expression and enzymatic cleavage of the mutated human protein with animal enzymes, do not meet the legal criteria of "anticipation" of the successful product of transgenic activity. A general recitation of known procedures, none of which was carried out by Mullan, does not defeat the "novelty" of the specific mouse that was actually produced by Elan.[4]

Elan states that the concluding clause of its claims, the processing of the human APP_{sw} to form detectable ATF-betaAPP in the rodent brain, is the "key element" of the claims. Elan stresses that the patent examiner required the inclusion of this limitation in order to distinguish the Mullan reference, for Mullan does not mention producing detectable ATF-betaAPP or its use as a proxy for detecting the smaller betaAP molecule. Elan argues that detection of the ATF-betaAPP permits determination of when the Swedish DNA has been successfully transferred and the mutated gene is

[4] In support of its argument on the uncertainty and difficulty of producing a successful transgenic mouse using known general procedures, Elan points out that the accused Mayo mouse was the 2,576th mouse that was screened.

successfully operating to produce the desired mutated protein and the desired enzymatic cleavage.

Mayo does not dispute that the Mullan reference makes no mention of the formation of ATF-betaAPP in detectable amounts in brain homogenate. Mayo argues, and the district court found, that this claim limitation is "inherent" in Mullan because a successful transgenic procedure and ensuing enzymatic cleavage will produce ATF-betaAPP. However, this was not shown by Mullan, and there was no evidence that the formation and detection of ATF-betaAPP in the transgenic mouse brain with the Swedish mutation was known to persons of ordinary skill in the field of the invention. Inherency cannot be based on the knowledge of the inventor; facts asserted to be inherent in the prior art must be shown by evidence from the prior art. Cf. In re Dembiczak, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) (criticizing the "hindsight syndrome wherein that which only the inventor taught is used against its teacher"). The purpose of the rule of inherency is to accommodate common knowledge, knowledge that judges might not know but that would be known to practitioners in the field. Finnigan Corp. v. Int'l Trade Comm'n, 180 F.3d 1354, 1365, 51 USPQ2d 1001, 1009 (Fed. Cir. 1999). On the law of anticipation, precedent has not improved on the words of Judge Learned Hand:

> No doctrine of the patent law is better established than that a prior patent or other publication to be an anticipation must bear within its four corners adequate directions for the practice of the patent invalidated. If the earlier disclosure offers no more than a starting point for further experiments, if its teaching will sometimes succeed and sometimes fail, if it does not inform the art without more how to practice the new invention, it has not correspondingly enriched the store of common knowledge, and it is not an anticipation.

Dewey & Almy Chemical Co. v. Mimex Co., 124 F.2d 986, 989 (2d Cir. 1942).

We conclude that the legal requirements of anticipation were not met. The summary judgment of invalidity based on anticipation is reversed, and the case is remanded for further proceedings.

C

Mayo states that the Elan position on infringement is that the claims of the patents in suit cover all transgenic mice with the Swedish mutation, and that if the claims are construed as broadly as Elan proposes, they are invalid under §103 or §112. These issues were not decided by the district court; they are not before us for review.

D

We respond to the remarks of our colleague in dissent, for he has inaccurately perceived the "ground" on which our decision "rests." The ground of our decision is, simply, that a novel patented product is not "anticipated" if it did not previously exist.

The dissenter objects to what he calls the patenting of "existing inventions." We too object to the patenting of existing inventions. However, Elan is not patenting something that previously existed, for Elan's mouse did not exist. While Mullan surely had the concept of creating a transgenic mouse with the mutated Swedish gene, as we have illustrated *ante*, Mullan did not make such a mouse and he did not tell (or know) which, if any, of the standard procedures from the scientific literature might be effective in achieving the complex series of transformations needed for a successful product. A general proposal to make a product that has not been made does not meet the criteria of "anticipation." Indeed, Mayo affirms in its brief that no mice had been made by Mullan; Mayo also affirms, contrary to the statements of the dissent, that "Mayo admits that some of the mice made according to the recipe [in the Mullan patent] will not have detectable ATF." Mayo brief at 19.

The dissent proposes that this decision will "have serious and unfortunate consequences in the future by permitting the securing of patent rights to existing inventions so long as the patent applicant identifies an inherent characteristic of that product that was not identified in the prior art," citing In re Cruciferous Sprout Litigation, 2002 U.S. App. LEXIS 17185 (Fed. Cir. 2002). We repeat, the Mullan mouse did not exist, quite unlike the broccoli sprouts of the Cruciferous Sprout Litigation, "long well known in nature and eaten by humans for decades." Id. at *5.

The dissenter appears to urge the unpatentability of any product that has been suggested but never made. This approach would eliminate even the possibility of patent protection for any transgenic product that may have been envisioned but not yet produced. A better rule is the established law, whereby new products are not "anticipated" when they did not previously exist, whether or not the process for making them is generally known. Although our colleague postulates "serious and unfortunate consequences in the future" if the Elan mouse is deemed patentable, others may believe that without the possibility of a patent on a new transgenic mouse, the hypothetical mouse envisioned by Mullan might well remain no more than a hypothesis. Determination of which consequence is fortunate or unfortunate is an important policy question; the law of anticipation as applied herein does not change existing policy.

"Anticipation" in the patent sense means that the subject matter was previously known. A precatory suggestion of general procedures that may or may not succeed in producing the novel product, a product that has not previously been produced, does not convert the suggested product into a previously existing product. The witnesses were in agreement that at the time the Mullan application was filed neither Mullan nor anyone else (1) had made a mouse harboring the Swedish mutated gene, (2) knew whether

the mouse DNA would accept the Swedish gene, (3) knew if the mouse cell would then express the human mutated protein of the Swedish family, or (4) knew whether the mouse enzymes would cleave the human mutated protein to produce human betaAP. Elan's expert Dr. Mobley stated, without disagreement, that "cells expressing the transgene have to correctly fold the protein, correctly modify it through glycosylation, correctly traffic it from internal to surface membranes, correctly traffic it through the endosomal pathway, and make it available to enzymes that modify it." Dr. Lieberburg, Elan's Chief Scientific and Medical Officer, stated that scientists were "at a great loss as to understand whether mice were even capable . . . of ever generating specific APP fragments that could be studied for drug discovery."

It is undisputed that Mullan had not made a mouse by any of his proposed procedures, and all of the scientists agreed that it cannot be predicted which, if any, procedure will ultimately succeed. General recipes of uncertain success do not convert a hoped-for product into one that previously existed. Our colleague in dissent states that despite Elan's statement that a successful mouse is a "tiny subset" of the transgenic mice that might be produced using Mullan's recipes, and despite the agreement of Mayo's witnesses with this scientific fact, the few successes that might be achieved (that is, that would possess the desired characteristics) form their own subset, thus placing the successful mouse in the prior art. That is not law of anticipation.

We observe the dissent's statement that an inventor's own disclosure can be used against him to prove anticipation. That statement is inaccurate. Patentability requires novelty and unobviousness in light of the prior art, not in light of what the inventor knew and included in his patent application. "Anticipation is the epitome of obviousness," Structural Rubber Products Co. v Park Rubber Co., 749 F.2d 707, 716. 223 USPQ 1264 1271 (Fed. Cir. 1984), and both are measured by what was previously known to persons in the field of the invention, as discussed in precedent. And as we have stated, the scope of the Elan claims was not decided, nor was it decided whether the Elan claims, upon correct construction, would cover the specific Mayo mouse. These issues are not before us on this appeal.

Finally, we note the dissent's observation that Elan's claims do not "require . . . a method of detection" of the ATF-beta APP. Elan has separate patents related to the method. See, e.g., U.S. Patent No. 5,441,870 (Method for monitoring cellular processing of β-amyloid precursor protein) to Seubert et al., claiming: "A method for monitoring cellular processing of β-amyloid precursor protein (β-APP) in cells, said method comprising detecting a soluble β-APP fragment secreted from said cells, and a substance which specifically binds to said soluble β-APP fragment, wherein the amino acid sequence of said β-APP fragment extends substantially from the amino-terminus of β-APP to the amino-terminus of β-amyloid peptide (β-AP)."

REVERSED AND REMANDED

United States Court of Appeals for the Federal Circuit

00-1467

ELAN PHARMACEUTICALS, INC.
AND ATHENA NEUROSCIENCES, INC.,

Plaintiff-Appellants,

v.

MAYO FOUNDATION FOR MEDICAL EDUCATION AND RESEARCH,

Defendant-Appellee,

DYK, Circuit Judge, dissenting.

The majority decision in this case rests upon the ground that an inventor's own disclosure may not be used under 35 U.S.C. § 102 as proof of anticipation by inherent disclosure in a prior art reference. This decision contradicts our own case law, which holds that knowledge of an inherent characteristic in the prior art is irrelevant. As we recently recognized in In re Cruciferous Sprout Litigation, No. 02-1031, slip op. at 12 (Fed. Cir. Aug. 21, 2002), on the issue of inherency "[i]t matters not that those of ordinary skill heretofore may not have recognized these inherent characteristics." Here, as in Cruciferous, while Elan "may have recognized something quite interesting about those [mice], it simply has not invented anything new." Id. at 13. This decision, if followed, will have serious and unfortunate consequences in the future by permitting the securing of patent rights to existing inventions so long as the patent applicant identifies an inherent characteristic of that product that was not identified in the prior art. That has never been our law. I respectfully dissent.

I

The patents asserted herein are U.S. Patent Nos. 5,612,486 ("the '486 patent") and 5,850,003 ("the '003 patent") (collectively "the Elan patents"). The sole independent claim of the '486 patent recites in relevant part:

> A transgenic rodent . . . comprising a transgene encoding a heterologous APP polypeptide having the Swedish mutation . . . wherein the transgene is expressed to produce a human APP polypeptide having the Swedish mutation, and wherein said polypeptide is processed to ATF-betaAPP in a sufficient amount to be detectable in a brain homogenate of said transgenic mouse.

Claim 1 of the '486 patent (emphases added). The sole independent claim of the '003 patent recites in relevant part:

> A transgenic rodent . . . comprising a transgene comprising . . . a DNA segment encoding a heterologous APP polypeptide . . ., wherein the transgene is expressed to produce a human APP polypeptide having the Swedish mutation, and wherein said polypeptide is processed to ATF-betaAPP in a sufficient amount to be detectable in a brain homogenate of said transgenic rodent.

Claim 1 of the '003 patent (emphases added). Because these claims are directed to transgenic rodents, the methods by which they are produced are not elements of the claims. Nor is there any claim to a method for detecting ATF-betaAPP.

Despite the clear language of the claims mandating their interpretation as products (transgenic rodents), much effort both at the district court and here on appeal has been expended on arguments incorrectly interpreting the claims in terms of methods. Elan argues, for example, that U.S. Patent No. 5,455,169 to Mullan (hereinafter "Mullan"), cited by the Mayo Foundation for Medical Education and Research (hereinafter "Mayo") as anticipating the claims of the Elan patents, fails to teach "how to detect ATF-betaAPP, much less how to detect the fragment in a brain homogenate." (Appellants' Br. at 23.) The claims of the Elan patents, however, require only detectable ATF-betaAPP and not a method of detection.

According to Elan, the '486 and '003 "patents required that its transgenic mice do all these things: [1] carry the APP_{SW} transgene, [2] express the APP_{SW} protein and [3] process the APP_{SW} to ATF-betaAPP such that the levels of ATF-betaAPP are detectable." (Appellants' Br. at 21.) As admitted by Elan in its brief on appeal, "Elan does not dispute that the specification of the Mullan patent disclosed a transgenic mouse harboring a human APP gene with the Swedish mutation." Id. at 17. In other words, the first element was disclosed. On appeal Elan also does not contend that the second element was not disclosed.[5] Elan contests solely the third aspect of the claims. Elan bases the novelty of its claimed rodents on the "critical element—processing APP to ATF-betaAPP in an amount sufficient to be detectable in a brain homogenate." Id. Mayo concedes that Mullan fails to expressly disclose this element of the claimed invention, but counters that this characteristic was inherent in the disclosure of Mullan. The only issue, therefore, is whether the rodent of Mullan will inherently produce ATF-betaAPP in a sufficient amount to be detectable in its brain homogenate.

II

On summary judgment the district court ruled that Mullan inherently anticipates the claims of the Elan patents, finding:

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[5] The majority appears to suggest that this element was not disclosed in Mullan, but this issue was not raised on appeal. In any event, the Mullan patent discloses the second element, stating "[a]s a result of the transfer, the resultant transgenic non-human animal will express one or more variant APP codon 670/1 polypeptides." Mullan, col. 11, ll. 34-36. The majority cites no authority suggesting that any more detailed description was required. To be sure the Mullan reference must have been enabling in this respect, In re Donohue, 766 F.2d 531, 533, 226 USPQ 619, 621 (Fed. Cir. 1985), and there may be a question as to whether it was enabling. But Elan has deliberately decided not to mount an enablement challenge to the Mullan patent, apparently for the reasons explained by the district court relating to potential for such arguments to invalidate Elan's own claims for lack of enablement. Elan Pharms., Inc. v. Mayo Found. for Med. Educ. & Research, 175 F. Supp. 2d 1209, 1212 (N.D. Cal. 2000).

> The mice claimed in the patents-in-suit are merely a subset of the mice described in Mullan. Some of the mice made using the process disclosed in Mullan (which is essentially the same process disclosed in the patents-in-suit) would inevitably have detectable levels of ATF-betaAPP. Were Plaintiffs to contend otherwise, their own patents would not be enabled. Mullan therefore inherently includes the [detectable ATF-betaAPP] limitation of the final "wherein" clauses of the asserted claims.

Elan Pharms., Inc., 175 F. Supp. 2d at 1212.

The majority disagrees, apparently because no extrinsic evidence of inherency existed in the prior art. The majority states:

> there was no evidence that the formation and detection of ATF-betaAPP in the transgenic mouse brain with the Swedish mutation was known to persons of ordinary skill in the field of the invention. Inherency cannot be based on the knowledge of the inventor; facts asserted to be inherent in the prior art must be shown by evidence from the prior art. Cf. In re Dembiczak, 175 F.3d 994, 999, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999) (criticizing the "hindsight syndrome wherein that which only the inventor taught is used against its teacher").

Ante at 11.

But this is not the correct analysis. This is not an obviousness case. The injunction in Dembiczak against using an inventor's own disclosure against him was in the context of a section 103 obviousness determination requiring "the oft-difficult but critical step of casting the mind back to the time of invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field." 175 F.3d at 999, 50 USPQ2d at 1617 (citing W.L. Gore & Assocs., Inc. v. Garlock, Inc., 721 F.2d 1540, 1553, 220 USPQ 303, 313 (Fed. Cir. 1983), cert. denied, 469 U.S. 851 (1984)). The perceived problem with combining references using hindsight to render a claimed invention obvious is that it "simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability." Id. This fear of hindsight recreation in the context of obviousness determinations, however, is not applicable in the context of inherency.

There is simply no basis in our law to support the proposition that the source of proof for inherency must be found in the prior art and cannot be found in a patentee's own disclosure or other source. In Continental Can Co. USA v. Monsanto Co., 948 F.2d 1264, 20 USPQ2d 1746 (Fed. Cir. 1991), the court noted that "[t]o serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence." Id. at 1268, 20 USPQ2d at 1749. Thus evidence extrinsic to the cited prior art reference may be used, i.e., the party raising the issue of inherency may fill in the gap in the disclosure using any source. The majority's contrary conclusion is incorrect as a matter of law, and directly contradicts our law, which has repeatedly recognized that the discovery of an inherent characteristic of an old product cannot be patented. Cruciferous, slip op. at 12; In re Spada, 911 F.2d 705, 709, 15 USPQ2d 1655, 1658 (Fed. Cir. 1990) ("When the claimed [inventions] are not novel they are not rendered patentable by recitation of properties, whether or not these properties are shown or suggested in the prior art." (emphasis added)); Titanium Metals Corp. of Am. v. Banner, 778 F.2d 775, 782, 227 USPQ 773, 779 (Fed. Cir. 1985) ("[I]t is immaterial, on the issue of their novelty, what inherent properties the [disclosed products] have or whether these applicants discovered certain inherent properties").

III

Because the disclosures of the Elan patents may be used as proof that the Mullan transgenic rodent inherently possessed the claimed characteristic, the remaining question is whether the Elan patents, in fact, provide that proof. They clearly do. The specification of the '003 patent teaches:

> Newly identified secreted fragments comprise amino-terminal portion of βAPP (Aβ) which remains after the cleavage and will be referred to hereinafter as the amino-terminal fragment form of βAPP

(ATF-ßAPP) [ATF-betaAPP]. ATF-ßAPP is believed to be the product of an alternative secretory processing pathway for Aß, which pathway is present even in normal (non-diseased) cells. It is further believed, however, that the alternate secretory pathway may be responsible for an essential event in the production of Aß in diseased cells in patients, and that abnormal production of AFT-ßAPP may be involved in diseases related to Aß plaque . . . .

Particularly preferred animal models for ß-secretase cleavage of Aß are transgenic animals which express the Swedish mutation of the Aß gene . . . . It has been found that such transgenic animals, particularly transgenic mice, produce high quantities of the AFT[sic ATF]-ßAPP which may [be] detected according to the methods of the present invention. In particular, it has been found that Swedish mutation of Aß produces quantities of the ATF-ßAPP which will usually be at least two-fold higher than wild type human ßAPP expressed in animals.

'003 patent, col. 12, ll. 21-42 (emphases added). The "discoveries" discussed in the preceding passage are two-fold: first, that the ß-secretase cleavage (metabolism) of the Swedish mutation form of APP to produce the ß-amyloid peptide (ßA) results in a secondary "newly identified" fragment, ATF-ßAPP; and second, that the newly discovered fragment is found in "high quantities" in transgenic mice having the Swedish mutation form of APP.

As Elan concedes on appeal, "the specification of the Mullan patent disclosed a transgenic mouse harboring a human APP gene with the Swedish mutation." (Appellants' Br. at 17.) More than simply "harboring" the gene as suggested by Elan, however, Mullan discloses a transgenic mouse that will express the gene to produce the Swedish APP and then metabolize the APP to produce the ß-amyloid peptide for the study of the underlying biochemistry of that metabolism. Mullan, col. 11, ll. 5-36 ("[S]uch model systems provide a tool for defining the underlying biochemistry of APP and ß-amyloid metabolism . . . . The human variant APP codon . . . can be transferred to a host non-human animal, such as a mouse. As a result of the transfer, the resultant transgenic non-human animal will express one or more variant APP codon 670/1 polypeptides."). As disclosed in the '003 specification, Swedish APP to ß-amyloid

metabolism directly produces the "newly identified" ATF-βAPP metabolite. '003 patent, col. 12, ll. 21-22. Further, transgenic mice that carry out the Swedish APP to ß-amyloid metabolism produce "high quantities" of the ATF-βAPP metabolite. Id. at col. 12, ll. 35-42. Because the claims are not limited to a particular "method of detection," but rather broadly recite the requirement that the fragments be "detectable," a mouse that metabolizes APP to produce the ß-amyloid peptide in sufficient amounts to permit the study of the underlying biochemistry of that metabolism would necessarily produce detectable amounts of the ATF-βAPP metabolite.

Elan argues that "[t]he transgenic mice claimed by Elan's patents are only a tiny, and at the time of Mullan unexpected, subset of the larger population of transgenic mice that might be produced by following the Mullan 'recipe.'" (Appellants' Reply Br. at 6.) In fact, the claimed mice are not a tiny subset of the mice disclosed in Mullan. To be sure, Mullan discloses a method for producing transgenic mice not all of which will successfully express the Swedish form APP. However, the Swedish form APP characteristic is disclosed in Mullan, and in each and every case of a mouse that processes Swedish form APP to produce the ß-amyloid chain as disclosed in the Mullan patent, that mouse will also produce ATF-βAPP as claimed in the Elan patents. '003 patent, col. 12, ll. 21-42. Thus, the rule that "[i]nherency . . . may not be established by probabilities or possibilities," In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA 1981) (citation omitted), is not violated by finding the claims of the Elan patent anticipated by a mouse according to Mullan (expressing and metabolizing the Swedish form APP), which will always possess the ATF-βAPP characteristic.

The district court correctly concluded that the claims of the Elan patents are invalid as inherently anticipated by Mullan.

For the foregoing reasons, I respectfully dissent.